A Haemophilus influenzae Gene That Encodes a Membrane Bound 3-Deoxy-β-manno-octulosonic Acid (Kdo) Kinase

POSSIBLE INVOLVEMENT OF KDO PHOSPHORYLATION IN BACTERIAL VIRULENCE*

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The lipopolysaccharide of Haemophilus influenzae contains a single 3-deoxy-β-manno-octulosonic acid (Kdo) residue derivatized with either a phosphate or an ethanolamine pyrophosphate moiety at the 4-OH position. In previous studies, we identified a kinase unique to H. influenzae extracts that phosphorylates Kdo-lipid IVₐ, a key precursor of lipopolysaccharide in this organism. We now designate this kinase Kdo kinase (22), is an enzyme that catalyzes the transfer of Kdo to lipid IVₐ (21). The transfer of Kdo to lipid IVₐ is essential for viability (7, 21). The E. coli Kdo transferase, encoded by the kdtA gene (22), is an unusual bi-functional enzyme (Fig. 1) that catalyzes the sequential addition of two Kdo residues in distinct glycosidic linkages to lipid IVₐ (23). Most other Gram-negative bacteria similarly contain at least two Kdo residues in their inner core (24). In H. influenzae, however, only a single Kdo is present.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) H10260.1.¶To whom correspondence should be addressed: Dept. of Biochemistry, Duke University Medical Center, Box 3711, Durham, NC 27710. Phone: 919-684-5326; Fax: 919-684-8885; E-mail: raetz@biochem.duke.edu.

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The first step of core biosynthesis in all Gram-negative bacteria is the addition of Kdo to the 6-OH of the precursor, lipid IVₐ (Fig. 1) (6, 10). In both E. coli and H. influenzae, the transfer of Kdo to lipid IVₐ is essential for viability (7, 21). The E. coli Kdo transferase, encoded by the kdtA gene (22), is an unusual bi-functional enzyme (Fig. 1) that catalyzes the sequential addition of two Kdo residues in distinct glycosidic linkages to lipid IVₐ (23). Most other Gram-negative bacteria similarly contain at least two Kdo residues in their inner core (24). In H. influenzae, however, only a single Kdo is present.

The abbreviations used are: LPS, lipopolysaccharide; Kdo, 3-deoxy-β-manno-octulosonic acid; PCR, polymerase chain reaction; kb, kilobase pairs; MALDI, matrix-assisted laser desorption/ionization.
(25–27). This Kdo is phosphorylated at its 4-OH position (17), the same site at which the second Kdo residue is attached in E. coli (Fig. 1).

By using a non-typeable strain of H. influenzae, we previously demonstrated that the H. influenzae Kdo transferase is mono-functional (28), i.e. capable of adding only a single Kdo residue to lipid IVA. In addition, we provided the first evidence for the presence of a Kdo kinase unique to extracts of H. influenzae (28) (Fig. 1). A homologue encoding a protein with 70% predicted similarity to E. coli KdtA was readily apparent by inspection of the H. influenzae genome (11). Analysis of the reaction product generated by the overexpressed recombinant H. influenzae KdtA confirmed this genomic assignment and the mono-functional activity of the protein. However, since no protein sequence was available for the Kdo kinase, the genome sequence alone was insufficient to permit identification of the kinase gene.

We now report the expression cloning and biochemical characterization of the Kdo kinase structural gene of H. influenzae. A cosmid library containing DNA fragments of the H. influenzae strain used for the genome project (Rd) (11) was constructed in E. coli. Lysates of single colonies harboring individual hybrid cosmids of the library were assayed for the presence of Kdo kinase activity, which is absent in E. coli (28) (Fig. 1). A single cosmid that directs the expression of the kinase was found. Interestingly, the gene encoding the kinase had previously been described as a possible open reading frame of unknown function, termed orfZ (7), in H. influenzae. Although OrfZ had been shown to be essential for virulence, no biochemical function could be assigned to the protein based solely upon its sequence and the apparently normal electrophoretic properties of the LPS isolated from orfZ mutants (7). In light of our discovery that orfZ encodes the Kdo kinase, the genetic and pathogenic studies by Hood et al. (7) can now be re-interpreted to suggest that the absence of Kdo phosphorylation in H. influenzae dramatically reduces virulence but does not stop bacterial growth. Given the identification of the biochemical function of orfZ, we suggest that the gene now be designated kdkA (for

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FIG. 1. Proposed reactions catalyzed by the Kdo transferase and the Kdo kinase of H. influenzae. Previous studies from our laboratory (28) have demonstrated that membranes of H. influenzae contain a mono-functional Kdo transferase and a unique Kdo kinase (lower half of the figure), neither of which is present in membranes of E. coli. The latter contain a bifunctional Kdo transferase (upper half of the figure) (21–23). The phosphate residue (red) incorporated by the Kdo kinase (KdkA) is proposed to be located at the Kdo 4-position, based on the most recent structural characterization of intact H. influenzae LPS (17). The structures of the mature lipid A (black) and Kdo (blue) domains of E. coli and H. influenzae lipopolysaccharides are shown at the right. These LPS substructures are the predominant species present in t-glycero-D-manno-heptose-deficient mutants of E. coli or H. influenzae (6, 26, 52). In the case of H. influenzae, an alternative molecular species (not shown) containing a phosphate residue at the 5- rather than the 4-position of Kdo was proposed in earlier studies by some authors (17, 26). However, in wild type LPS of both E. coli and H. influenzae, the first heptose residue of the core (not shown) is attached to the 5-OH position of the inner Kdo (6, 8, 17). In E. coli the substituent X denotes partial substitution with a phosphate residue (53, 54), whereas in H. influenzae Y denotes partial substitution with an ethanolamine phosphate moiety (7, 17). The LPS substructures found in heptose-deficient mutants (above right) are sufficient to support bacterial growth, but such mutants are not virulent (7).
**TABLE I**

| Bacterial strain or plasmid | Relevant genotype | Source               |
|-----------------------------|------------------|----------------------|
| H. influenzae, strain 722   | Non-typeable wild type |                     |
| H. influenzae, strain Rd    | Wild type        | 28                   |
| XL1 Blue-MR                 | ΔmcrABC, recA1, lac | ATCC (51907)         |
| Sure                       | lac-Δ2ΔM15, Tet'  | Stratagene           |
| BLRD3E/pLysS               | Δor1-recA506:7Δ10DE3, Tet'/Cm' | Stratagene |
| B67E                       | XL1 Blue-MR containing cosmid TE | This work |

**Plasmids**

| pWE15         | Cosmid, Amp' | Stratagene |
| pBluescript K2 | lacZ, Amp'   |            |
| pET21a        |             | Novagen    |
| pE3.2         | pWE15 encoding the Kdo kinase activity (kdkA/orfZ), Amp' | This work |
| pB6A         | 4556-bp EcoRI fragment cloned into pBluescript, kdkA', Amp' | This work |
| pBl100       | 1192-bp BamHI deletion from pE3.2, kdkA', Amp' | This work |
| pET21a        | Intergenic region between orfM and opsX cloned into pBluescript, kdkA', Amp' | This work |
| pKdkA        | Vector containing a T7 promoter, Amp' | This work |
| pE21a        | pET21a containing Kdo kinase (orfZ) coding region, Amp' | This work |

“Kdo kinase A)” (Fig. 1). Consistent with the occurrence of phosphorylated Kdo residues in some Gram-negative bacteria (29–32), significant homologues of kdkA are present in _Bordetella pertussis_, _Vibrio cholerae_, _Actinobacillus actinomycetemcomitans_, _Shewanella putrefaciens_, and _Pateurella multocida_.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP was purchased from NEN Life Science Products. Kdo, HEPES, EDTA, EGTA, NAD+, heme, CTP, ATP, and other nucleotides were purchased from Sigma. Triton X-100 was Surfact-Amps grade from Pierce. Yeast extract, tryptone, and brain heart infusion were obtained from Difco. All other chemicals and solvents were reagent grade. DEAE-cellulose (DE52) was purchased from Whatman. The 0.25-mm glass backed Silica Gel 60 thin layer chromatography plates were from Merck.

**Bacterial Strains and Growth Conditions**—The various strains and plasmids utilized for the experiments described are detailed in Table I. _H. influenzae_ strain Rd (catalog number 51907) was purchased from ATCC. The _H. influenzae_ cells were grown at 37 °C in brain heart infusion medium (37 g/liter) supplemented with heme (10 µg/ml) and NAD+ (10 µg/ml) (33). _E. coli_ strains were grown at 37 °C on Luria broth, consisting of 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter (34). When applicable, the cultures were supplemented with 50 µg/ml ampicillin and/or 10 µg/ml chloramphenicol.

**Preparation and Isolation of Substrates**—Milligram quantities of the precursor lipid IV₇ were prepared as described previously (35). Prior to use in assays and the synthesis of Kdo-lipid IV₇, the lipid was subjected to reverse phase chromatography (36). Unlabeled Kdo-lipid IV₇ was prepared by a previously described method (28). Both unlabeled and Kdo-[32P]lipid IV₇ were prepared by the published methods (37, 38). The [4-32P]lipid IV₇ was prepared by the method of Brozek et al. (39), using membranes isolated from the _E. coli_ strain pJK2/BLR(DE3), which overexpresses the 4'-kinase (40). The Kdo[4-32P]lipid IV₇ was synthesized by a slight modification of the published method (28). Briefly, [4-32P]lipid IV₇ was prepared as usual (38), but at the end of the reaction, the volume was adjusted to 180 µl with water. The solution was then converted to a two-phase Blish/Dyer system, consisting of CHCl₃/methanol/H₂O (2:2.1:8, v/v/v) (41, 42), by the addition of 200 µl of both CHCl₃, and methanol. The tube was mixed thoroughly and centrifuged at 20,800 × g for 5 min at room temperature to separate the phases. The chloroform-rich lower phase was removed and transferred to a fresh tube. The upper phase was then washed twice with pre-equilibrated acetic acid lower phase, i.e., a lower phase generated by mixing chloroform/methanol/0.1 M HCl (2:2:1.8, v/v). The resulting lower phases were pooled with the initial lower phase and dried under a stream of nitrogen. The reaction components for the mono-functional Kdo transferase reaction (28) were then added to the tube. Following the Kdo transferase reaction, the Kdo[4-32P]lipid IV₇ was isolated by preparative thin layer chromatography as described (28). All lipids were stored as aqueous dispersions at −20 °C and were dispersed again after thawing by sonic irradiation in a bath for 30–60 s prior to use. Recombinant _E. coli_ CMP-Kdo synthetase (43) was partially purified as described by Brozek et al. (37).

**Recombinant DNA Techniques**—_H. influenzae_ Rd genomic DNA was prepared as described previously (33). Plasmid DNA was isolated using the Qiagen Mini-Prep purification system (Qiagen). Restriction endonucleases (New England Biolabs), T4 DNA ligase (Life Technologies, Inc.), and shrimp alkaline phosphatase (U. S. Biochemical Corp.) were used according to the manufacturers’ instructions. DNA sequencing was performed at the Duke University Medical Center shared DNA sequencing facility.

**Kdo Transferase Assay**—Kdo transfer from the donor, CMP-Kdo, to the acceptor, [4-32P]lipid IV₇, was assayed as described previously (28). The reaction mixtures (typically 10–20 µl) contained 50 mM HEPES, pH 7.5, 2 mM Kdo, 0.1% Triton X-100, 100 µM [4-32P]lipid IV₇ (3000–6000 cpm/nmol), 5 mM CTP, 10 mM MgCl₂, and 1.8 milliliters of partially purified, recombinant CMP-Kdo synthase. Assays (at 30 °C) were initiated by the addition of enzyme, usually _H. influenzae_ membrane preparations, as indicated. The reactions were terminated by spotting 5-µl portions onto a thin layer plate. The plate was dried under a cool air stream and developed in the solvent chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v). The solvent was evaporated with a hot air stream, and the plate was exposed to a PhosphorImager screen for 12–16 h. The conversion of [32P]-labeled substrate to product was quantified using a Molecular Dynamics PhosphorImager equipped with ImageQuant software.

**Kdo Kinase Assay**—The Kdo kinase was assayed using the acceptor Kdo[4-32P]lipid IV₇, as described previously (28). The conditions were very similar to those used for the Kdo transferase. Briefly, reaction mixtures (10–20 µl) contained 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 100 µM [4-32P]lipid IV₇ (3000–6000 cpm/nmol). The reactions were initiated with enzyme and incubated for designated times at 30 °C. The assays were terminated, and the substrate and product were resolved by thin layer chromatography, as described above for the Kdo transferase. When membranes from pKdkA/BLR(DE3)/pLysS were used in the assays, 1 mg/ml bovine serum albumin was included in the reaction mixture. Other minor modifications to the standard reaction conditions are noted in the figure legends.

**Construction of H. influenzae Rd Genomic Library**—A cosmid library of _H. influenzae_ strain Rd (11) was constructed in _E. coli_ XL1-Blue MR (Strategene), utilizing the Gigapack III XL packaging system (Strategene). Briefly, 100 µg of genomic DNA was partially digested with _Sau3A1_ (New England Biolabs) until the predominant DNA fragments were approximately 20 kb in size, as judged by agarose gel electrophoresis. The fragments were then ligated into the cosmid pWE15 (Strategene). Prior to ligation, pWE15 was digested with _BamH_I, gel-purified (Qiagen Gel Extraction Kit), and treated with phosphatase, according to standard procedures (44). The ligation mixtures were then packaged into recombinant λ phage using the Gigapack III XL packaging system. To determine the colony-forming units per µl, the packaging extracts were titered using strain XL1 Blue-MR. The library was subsequently amplified in XL1 Blue-MR, and aliquots were frozen as glycerol stocks at −80 °C.

**Preparation of Cosmid Library Lysates and Initial Screening for Kdo Kinase Expressing Clones**—Individual colonies from the library were grown in microtiter plates, and lysates were prepared by the method ofDotson et al. (45). A portion of the amplified library was thawed and diluted appropriately (1:1 × 10⁶). Then, 100-µl portions of the diluted library were plated onto LB agar plates containing 50 µg/ml ampicillin. The plates were incubated overnight at 37 °C. Single colonies were picked from these plates and used directly to inoculate six 96-well
microtitr dishes (containing 150 µl of LB medium with ampicillin per well). The dishes were placed in an air shaker and incubated overnight at 37 °C. The overnight dishes were used to inoculate fresh microtitr plates (containing 200 µl of LB medium with ampicillin per well) using a sterile 96-prong apparatus (Nalge Nunc International). Sterile glycercol (5%) was added to the overnight plates (to a final concentration of 20%). After mixing, the plates were frozen at −80 °C for later use. Meanwhile, the freshly inoculated plates were grown for 6 h at 37 °C with rotary shaking at 200 rpm. The cultures were then centrifuged at 3,600 × g for 20 min at 4 °C, and the supernatants were decanted. The microtitr plates were placed on ice, and the cell pellets were resuspended in 25 µl of 33 mM Tris-HCl, pH 8.0. Following resuspension, the Tris-HCl 33 mM containing 0.2% Triton X-100, 0.5 M EDTA was added to each well. The plates were incubated for 5 min on ice and then frozen at −80 °C. Just prior to assay, the cells were lysed by thawing the plates at room temperature for 10 min.

The lysates of the cells containing the individual cosmid were assayed by the Kdo kinase assay of White et al. (28) with slight modifications. After expressing the Kdo kinase from the pIB100 clone, the 25 ml of lipodIva was centrifuged at 150,000 g for 60 min at 4 °C. The supernatants were decanted, and the pellet was resuspended in buffer (containing 100 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1% Triton X-100, 100 µM nuclease-free bovine serum albumin, 20 µM each dNTP, 2 mM MgCl₂, and 2.5 units of Pfu DNA polymerase (Stratagene) in a final volume of 0.05 ml). The mixture was subjected to 5 min of denaturation at 94 °C and then 25 cycles of 94 °C for 1 min, 50 °C for 1.5 min, 72 °C for 1 min, and ended with a 7-min extension at 72 °C in a Perkin-Elmer GeneAmp PCR system 2400. The PCR product was digested with HindIII and BamHI, and ligated into pBlueScript IKS, which had been digested similarly. A portion of the ligation mixture was transformed into Sure cells (Stratagene). Plasmid containing colonies were selected as described above. The insert in pIB100 was confirmed by DNA sequencing.

Construction of Plasmid (pKdkA) with the orfZ/kdkA Gene Bounded by the T7 Promoter—To overexpress the Kdo kinase to high levels, the orfZ/kdkA gene was cloned into pET21a (Novagen), under the control of the T7 promoter. The gene was amplified by PCR using pE3.2 as the template. The primers were as follows: the forward primer, 5′-GCC CGC CAT ATG CAC CAA TTC CTC 3′, and the reverse primer, 5′-GCC CGG ATC CGC TTT TAT TGA TGG 3′. The forward primer introduces a NdeI restriction site (shown in bold) at the start codon of the T7 promoter, and the reverse primer creates a BamHI site downstream of the stop codon of the gene. The PCR product was digested with NdeI and BamHI and ligated into pET21a cut with the same enzymes. The ligation mixtures was used to transform Sure cells, as described above. The presence of the appropriate insert was confirmed by restriction digestion of the plasmid with NdeI and BamHI. The desired plasmid was designated pKdkA. The insert in pKdkA was confirmed by DNA sequencing. For overexpression of active KdkA, plasmid was transformed into BL21(DE3)pLYSs (Novagen).

Large Scale Preparation of Phospho-Kdo-Lipid IVα—The Kdo kinase reaction was optimized so that the substrate, Kdo-lipid IVα, was converted to a single phase Bligh/Dyer system by the addition of 1.24 ml of CHCl₃ and 0.263 ml of H₂O per ml of supernatant. After mixing, the mixture was centrifuged at 9,000 g for 15 min at 4 °C. The supernatants were divided between two fresh Corex 125 mm borosilicate tubes. The reaction mixtures were incubated at 94 °C and then 25 cycles of 94 °C for 1 min, 50 °C for 1.5 min, 72 °C for 1 min, and ended with a 7-min extension at 72 °C in a Perkin-Elmer GeneAmp PCR system 2400. The PCR product was digested with HindIII and BamHI, and ligated into pBlueScript IKS, which had been digested similarly. A portion of the ligation mixture was transformed into Sure cells (Stratagene). Plasmid containing colonies were selected as described above. The insert in pIB100 was confirmed by DNA sequencing.

Expression Cloning a Kdo Kinase Gene from H. influenzae—Expression cloning was carried out using the pLysS LB/BL21(DE3)/pLysS and BLR(DE3)/pLysS. The mixtures were incubated at 30 °C for 15 min and then centrifuged at 9,000 g for 15 min at 4 °C. The supernatants were divided between two fresh Corex 125 mm borosilicate tubes. The reaction mixtures were incubated at 94 °C and then 25 cycles of 94 °C for 1 min, 50 °C for 1.5 min, 72 °C for 1 min, and ended with a 7-min extension at 72 °C in a Perkin-Elmer GeneAmp PCR system 2400. The PCR product was digested with HindIII and BamHI, and ligated into pBlueScript IKS, which had been digested similarly. A portion of the ligation mixture was transformed into Sure cells (Stratagene). Plasmid containing colonies were selected as described above. The insert in pIB100 was confirmed by DNA sequencing. The presence of the appropriate insert was confirmed by restriction digestion of the plasmid with NdeI and BamHI. The desired plasmid was designated pKdkA. The insert in pKdkA was confirmed by DNA sequencing. For overexpression of active KdkA, plasmid was transformed into BL21(DE3)pLYSs (Novagen).

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DEAE-cellulose column (Whatman DE52), equilibrated as the acetate form in the same solvent (35). The column was then washed with 6 ml of CHCl₃/methanol/120 mM aqueous ammonium acetate (2:3:1, v/v), followed by 6 ml of CHCl₃/methanol/240 mM aqueous ammonium acetate (2:3:1, v/v) and 6 ml of CHCl₃/methanol/0.5 M aqueous ammonium acetate (2:3:1, v/v). The lipid product, phospho-Kdo-lipid IVₐ, was eluted with ~30 ml of CHCl₃/methanol/1 M aqueous ammonium acetate (2:3:1, v/v). Fractions containing the product were identified by spotting 5 μl of each fraction onto a silica thin layer chromatography plate, which was developed in the same solvent system described above for the Kdo kinase assay. The lipid was detected by spraying the dried TLC plate with 25% sulfuric acid in ethanol, followed by charring on a hot plate. Fractions from the DEAE column containing phospho-Kdo-lipid IVₐ were pooled and were converted to a two-phase Bligh-Dyer system by the addition of 0.17 ml of CHCl₃ and 0.28 ml of H₂O per ml of pool. After thorough mixing in 25-ml Corex tubes, the phases were separated by centrifugation at 1,900 × g for 20 min. The combined upper phase (~20 ml) was washed three times with ~10 ml of neutral pre-equilibrated lower phase (prepared as described above). The lower phases were pooled, and 5–10 μl of high pressure liquid chromatography grade pyridine was added, and the solvent was removed by rotary evaporation at room temperature. The pure phospho-Kdo-lipid IVₐ (~2–3 mg) was stored dry at −20 °C until further analysis.

Mass Spectrometry of the Product Generated by the Recombinant Kdo Kinase—Spectra were acquired in the negative-ion linear mode by using a Kratos Analytical (Manchester, UK) time of flight matrix-assisted laser desorption/ionization (MALDI) mass spectrometer, equipped with a 337 nm laser, a 20-kV extraction voltage, and time-delayed extraction (47). Each spectrum was the average of 50 shots. The matrix was a mixture of saturated 6-aza-2-thiophenolate in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v). The product generated by the recombinant Kdo kinase was dissolved in a mixture of chloroform/methanol (4:1, v/v) before being mixed with the matrix (1:1, v/v) on a slide. The sample mixtures were allowed to dry at room temperature prior to mass analysis. The hexa-acetylated lipid A 1,4'-bis-phosphate from E. coli K-12 (Sigma) was used as an external mass standard.

RESULTS

Kdo Transferase and Kdo Kinase Activities in Membranes of H. influenzae Rd—Before constructing a genomic library, it was necessary to confirm that H. influenzae Rd membranes possessed the same Kdo transferase and Kdo kinase activities previously observed in strain 722 (28). Therefore, membranes from H. influenzae Rd and 722 were assayed in parallel for these enzymatic reactions (Fig. 2). The addition of only a single Kdo residue to [4-³²P]lipid IVₐ was observed with both types of membranes (Fig. 2, lanes 2 and 3). The calculated specific activity of Kdo transfer by the Rd membranes was about the same as that seen with strain 722 membranes (1.7 and 2.4 nmol/min/mg, respectively).

The Kdo kinase is assayed with purified Kdo[4'-³²P]lipid IVₐ as the acceptor and unlabeled ATP the donor. The addition of the hydrophobic phosphate group to Kdo[4'-³²P]lipid IVₐ generates a product that migrates more slowly than the substrate. Comparable kinase activities are present in both the Rd and the 722 membranes (Fig. 2, lanes 5 and 6), and the specific activities are 9.6 and 8.3 nmol/min/mg, respectively.

Screening of a H. influenzae Rd Genomic Library for a Cosmid That Directs Expression of Kdo Kinase in E. coli—A cosmid library was constructed in E. coli XL1-MR using genomic DNA fragments of H. influenzae Rd as inserts in the cosmid vector pWE15. To determine the approximate sizes of the genomic inserts, 10 random single colonies were picked, and the cosmid from each colony was isolated. The cosmid DNAs were digested with EcoRI, labeling the inserted DNA from the vector. The insert sizes, estimated by gel electrophoresis, ranged from 10 to 20 kb (not shown).

Considering the size of the H. influenzae genome (1.8 megabases) (11) and the average sizes of the DNA inserts in the cosmid library, 472 individual colonies were initially picked from the cosmid library to generate a set of lysates suitable for screening for the expression of Kdo kinase activity. The lack of Kdo kinase in E. coli (28) made it a convenient background with which to search for this H. influenzae gene. However, in the concentrated lysates prepared in microtiter plates, some conversion (1–4%) of the Kdo[4'-³²P]lipid IVₐ to an unidentified product migrating like phospho-Kdo[4'-³²P]lipid IVₐ was observed irrespective of which DNA insert was present (Fig. 3). The nature of this background reaction was not characterized, as only those extracts capable of generating higher levels of phospho-Kdo[4'-³²P]lipid IVₐ-like material were of interest (Fig. 3). Accordingly, out of the 472 colony lysates, 10 possible candidates were identified for further evaluation, including the seven active extracts shown in Fig. 3.

Variability in protein concentrations, in conjunction with the ATP-independent modification(s) of Kdo-lipid IVₐ seen in the concentrated colony lysates (Fig. 3), necessitated the re-assay of the 10 active colonies under more controlled conditions. Larger cultures (100 ml) were grown to A₆₀₀ = −0.9 from the colonies harboring each of the candidate cosmids, and French press extracts were prepared. By using a final protein concentration of 0.5 mg/ml in each assay, the formation of phospho-Kdo[4'-³²P]lipid IVₐ and the ATP dependence of the reaction was re-evaluated for the 10 Kdo kinase candidates. The results for the seven active clones from Fig. 3 are shown in Fig. 4. The XL1 control samples (Fig. 4) illustrate the lack of Kdo kinase activity in extracts of the E. coli host strain. Only clone 7E

FIG. 2. Demonstration of a mono-functional Kdo transferase and a Kdo kinase in H. influenzae strains 722 and Rd. Membranes were assayed for either Kdo transferase (lanes 1–3) or Kdo kinase (lanes 4–6). Assays were performed under the standard conditions described under "Experimental Procedures." In each reaction, 100 μM [4-³²P]labeled lipid acceptor was used as the substrate, as indicated. The reactions were incubated for 20 min at 30 °C. For the Kdo transferase reactions, lane 1 is the no enzyme control. Lane 2 demonstrates the mono-functional Kdo transferase activity in membranes (0.5 mg/ml) of H. influenzae strain 722 (28), and lane 3 shows the comparable mono-functional Kdo transferase activity in Rd membranes (0.5 mg/ml). Lanes 4–6 are the Kdo kinase assays. Lane 4 is the no enzyme control. Lanes 5 and 6 demonstrate the ATP-dependent phosphorylation of Kdo[4'-³²P]lipid IVₐ catalyzed by strain 722 and strain Rd membranes, respectively (each at 0.05 mg/ml).
In the absence or presence of 5 mM ATP, as indicated. The reactions ATP dependence of the reaction was assessed by assaying the extracts were incubated for 30 min at 30 °C and analyzed by TLC analysis to determine the extent of phospho-Kdo[4-32P]lipid IVA formation.

Localization of the Kdo Kinase Gene—Cosmid cloning identified the region encoding the Kdo kinase in H. influenzae genome (11). The open reading frames located within this region are shown in Fig. 5. Both lgtC and hemR were ruled out as candidates for the kinase gene, since their sequences were partially deleted in pE3.2. Construction of the active subclone pB6A (Fig. 5) similarly eliminated orfM.

The only complete open reading frames on pB6A were orfZ (7) (a gene of unknown function also recently designated HI0260.1) and opsX (7, 11) (HI0260, thought to encode heptosyltransferase I of H. influenzae). Therefore, pIB100, was constructed by PCR (Fig. 5) to examine the function of orfZ. Transformation of pIB100 into Sure cells and assay of extracts demonstrated that orfZ and its immediate flanking DNA could indeed direct the expression of the kinase (not shown). The orfZ gene (Fig. 6) encodes for a protein of 241 amino acids. The sequence of the pIB100 insert (Fig. 6) is 100% identical to that in the H. influenzae genomic data base (11). Assay of pIB100/Sure cell membranes and cytosol demonstrated that ~80% of the Kdo kinase activity is localized to membranes (not shown), as seen with the wild type enzyme (28). However, hydropathy analysis of OrfZ revealed no obvious membrane spanning regions (not shown).

Although not initially designated as an open reading frame in the genome project, the interval containing orfZ was considered by Hood et al. (7) as a possible gene involved in LPS biosynthesis, since it is located between the core glycosyltransferase genes, lgtC and opsX (Fig. 5). No function could be ascribed to orfZ based on its sequence, and the LPS isolated from mutants containing insertions in orfZ did not appear to be dramatically altered (7). Given its function as the Kdo kinase gene, we propose the new designation kdkA in place of orfZ.

Overexpression of the Kdo Kinase and Characterization of the Recombinant Enzyme—Once the identity of the Kdo kinase gene was established, the plasmid pKdkA was constructed in which the kinase gene was placed behind a T7 promoter. Next, pKdkA was transferred into E. coli BLR(DE3)/pLysS, which synthesizes T7 polymerase when induced with isopropyl-β-D-thiogalactopyranoside. Following induction, the Kdo kinase was greatly overproduced, representing about 60% of the total membrane protein, as judged by SDS gel electrophoresis and Coomassie Blue staining (not shown). The specific activity of the overexpressed kinase in membranes was about 70,000 nmol/min/mg, ~8,000-fold higher than in wild type H. influenzae Rd membranes (8.6 nmol/min/mg). Membranes isolated from control cells harboring the vector without the insert contained no measurable Kdo kinase activity.

The membranes isolated from induced cells of pKdkA/
BLR(DE3)/pLysS were used to characterize some of the catalytic properties of the Kdo kinase. As seen with wild type H. influenzae membranes, the recombinant kinase activity was optimal in the presence of 0.1–0.2% Triton X-100 and had maximal activity at a pH of 7.5 in HEPES buffer (not shown). The reaction was linear with membrane protein from 0.02 to 0.4 mg/ml and with time for up to 10 min at 0.02 mg/ml (Fig. 7).

The apparent \( K_m \) for Kdo-lipid IVA at saturating ATP concentration (5 mM) was 11.6 ± 0.8 mM, with a \( V_{max} \) of 73,625 nmol/min/mg (at a protein concentration of 0.04 mg/ml).

Like the wild type H. influenzae kinase, the recombinant Kdo kinase in membranes of pKdkA/BLR(DE3)/pLysS preferred ATP over other nucleotide triphosphates (Fig. 8) (28). However, minor enzymatic activity was detected with GTP (Fig. 8). Furthermore, as shown in Fig. 8, the Kdo kinase displays an absolute requirement for Mg\(^{2+}\).

Detection of the Kdo Kinase with \([\gamma-^32P]\)ATP as the Donor—Under our standard conditions for assaying the Kdo kinase, the acceptor is Kdo[4\(^{32}^P\)]lipid IV\(_A\) and the donor is unlabeled ATP. To confirm that the observed reaction product is indeed phospho-Kdo[4\(^{32}^P\)]lipid IV\(_A\) (not an alternative product such as an adenylated derivative of Kdo[4\(^{32}^P\)]lipid IV\(_A\)), \([\gamma-^32P]\)ATP was utilized as the donor and unlabeled Kdo-lipid IV\(_A\) as the acceptor. The reactions were then analyzed by thin layer chromatography, followed by PhosphorImager analysis, to demonstrate the incorporation of \(^32P\) into the putative phospho-Kdo-lipid IV\(_A\) (Fig. 9).

In lanes 4–6 of Fig. 9, the substrate concentrations are the same as in lanes 1–3, but \([\gamma-^32P]\)ATP is used in conjunction with unlabeled lipid acceptor. Lane 4 (the no enzyme control) shows the migration of the \([\gamma-^32P]\)ATP substrate in this solvent system. Lane 5 is derived from a reaction mixture containing the recombinant kinase and \([\gamma-^32P]\)ATP but lacking Kdo-lipid IVA. In this case, no \(^32P\)-labeled lipid product is formed. Finally, lane 6 of Fig. 9 conclusively demonstrates that the \(^32P\) of \([\gamma-^32P]\)ATP is transferred to Kdo-lipid IV\(_A\) when all components of the system are present. The lipid product obtained in this manner (Fig. 9, lane 6) migrates with the same RF as that obtained with Kdo[4\(^{32}^P\)]lipid IV\(_A\) and unlabeled ATP (Fig. 9, lane 3).
Expression Cloning a Kdo Kinase Gene from H. influenzae

Lipid Acceptor Specificity of the Kdo Kinase—To localize the region of the Kdo[lipid IV A molecule to which the phosphate group is transferred, the lipid acceptor specificity of the Kdo kinase reaction was examined. Three related compounds were used as follows: [4'-32P]lipid IV A, Kdo[4'-32P]lipid IV A, and Kdo₂[4'-32P]lipid IV A, as indicated. All the lipids were added at 10 μM (6,000 cpm/nmol). The reactions were initiated with 0.016 μg/ml membranes prepared from pKdkA/BLR(DE3)/pLysS and were incubated for 2 min at 30 °C. The only lipid that functioned as an acceptor substrate was Kdo[4'-32P]lipid IV A.

Mass Spectrometry of the Product Generated by the Recombinant Kdo Kinase—The reaction product generated by the recombinant Kdo kinase was isolated and subjected to mass spectrometry. The MALDI-time of flight mass spectrum in the negative mode is shown in Fig. 11. The structure of the proposed phospho-Kdo[lipid IV A product is shown in the inset. The prominent peak at m/z 1705.1 is interpreted as the (M-H)²⁺ of the parent compound, given the predicted molecular weight of 1705.86 for phospho-Kdo[lipid IV A. The observed (M-H)²⁺ confirms that only a single phosphate group is transferred to Kdo[lipid IV A by the recombinant kinase. The other major peak at m/z 1405.2 represents the anionic lipid fragment remaining after cleavage of the Kdo glycosidic linkage, consistent with the loss a phospho-Kdo unit from the parent compound. This lipid fragment is interpreted as the lipid IV A anion, which has a predicted molecular weight of 1404.7. The observed fragmentation pattern is very similar to that reported in previous studies with H. influenzae membranes (28) and further validates the proposal that the kinase phosphorylates the 4-OH of the inner Kdo.

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In the present study we report the first identification and cloning of a structural gene encoding a Kdo kinase (28) (Fig. 1). The H. influenzae gene that we have found corresponds to a previously identified open reading frame, designated orfZ (or HI0260.1) (7). Hood and co-workers (7) postulated that orfZ might be involved in H. influenzae LPS biosynthesis because of its proximity to two other genes (opsX and igtC) encoding putative core glycosyltransferases (Fig. 5). However, they were unable to assign a biochemical function to the orfZ gene because of its unique sequence and the apparently normal size of the LPS isolated from orfZ mutants (7). Our previous discovery and development of an assay for the Kdo kinase (28) in extracts of H. influenzae have now enabled the unambiguous identification of orfZ as the Kdo kinase structural gene. Expression cloning, in conjunction with the availability of the H. influenzae genomic data base (11), greatly accelerated the search for the kinase gene. As illustrated by our study, new biochemical assays will be very useful for elucidating the roles of the many previously uncharacterized (or partially characterized) natural products like LPS (6, 48) or the identification of novel physiological processes.

The cloning and overexpression of kdkA facilitated the characterization of several catalytic properties of the kinase that previously were difficult to evaluate using H. influenzae membranes (28) as the enzyme source. Specifically, the divalent cation requirement (Fig. 8) and the incorporation of 32P from [γ-32P]ATP into the lipid product (Fig. 9) were demonstrable with the overexpressed kinase. Mass spectrometry (Fig. 11) confirmed the incorporation of only a single phosphate residue by the recombinant Kdo kinase. However, the proposed location of the phosphate group at position 4-OH on the Kdo moiety remains to be confirmed.

The conclusive identification of the Kdo kinase structural gene has allowed for the search for related genes in other Gram-negative bacteria. Highly significant kdkA homologues were found in the partially sequenced genomes of B. pertussis, V. cholerae, A. actinomycetemcomitans, S. putrefaciens, and P. multocida. Strains of B. pertussis and V. cholerae contain a phosphate group attached to the single Kdo that is present in their LPS (29–32), and Actinobacillus is closely related to H. aphrophilus. A distant homologue of kdkA is also present in P. gingivalis, which contains a single Kdo in its LPS, thought to be phosphorylated at position 7 or 8 (49). 

The biological significance of Kdo phosphorylation versus the presence of multiple Kdo residues that are characteristic of the inner LPS cores of most other Gram-negative bacteria remains to be established. Based on our findings, it appears that Kdo phosphorylation in H. influenzae LPS plays a crucial role in modulating the virulence of the bacteria in certain animal models. Our identification of kdkA/orfZ as the structural gene for the Kdo kinase sheds new light on the intriguing genetic and biochemical data of Hood et al. (7), who found that when kdkA/orfZ was disrupted, the bacteria were viable and made LPS of relatively normal size but were rendered avirulent. Apparently, Kdo phosphorylation is somehow essential for bacterial pathogenesis. It would now be very interesting to introduce the bifunctional E. coli transferase (22) into a H. influenzae mutant deficient in kdkA and determine whether or not the addition of a second Kdo in place of the phosphate can restore bacterial virulence.

As a next step, it will be important to demonstrate that the Kdo kinase is indeed missing in the orfZ-deficient mutants reported by Hood et al. (7). The absence of the Kdo-linked phosphate group in the LPS of this mutant in vivo also remains to be confirmed. We cannot yet exclude the scenario that H. influenzae contains more than one Kdo kinase, although this seems unlikely based on the genome sequence (11). Finally, the possibilities that a defect in Kdo phosphorylation causes secondary changes in LPS structure, outer membrane protein assembly, and/or growth rate need to be investigated before the biological significance of Kdo phosphorylation in pathogenesis can be fully evaluated.

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