The lipopolysaccharide (LPS) of the deep rough mutant *Haemophilus influenzae* I69 consists of lipid A and a single 3-deoxy-D-manno-oct-2ulosonic acid (Kdo) residue substituted with one phosphate at position 4 or 5 (Helander, I. M., Lindner, B., Brade, H., Altmann, K., Lindberg, A. A., Rietschel, E. T., and Zähringer, U. (1988) *Eur. J. Biochem.* 177, 489–492). The *waaA* gene encoding the essential LPS-specific Kdo transferase was cloned from this strain, and its nucleotide sequence was identical to *H. influenzae* DSM11121. The gene was expressed in the Gram-positive host *Corynebacterium glutamicum* and characterized in vitro to encode a monofunctional Kdo transferase. *waaA* of *H. influenzae* could not complement a knockout mutation in the corresponding gene of an *Escherichia coli* strain. However, complementation was possible by coexpressing the recombinant *waaA* together with the LPS-specific Kdo kinase gene (*kdkA*) of *H. influenzae* DSM11121 or I69, respectively. The sequences of both *kdkA* genes were determined and differed in 25 nucleotides, giving rise to six amino acid exchanges between the deduced proteins. Both *E. coli* strains which expressed *waaA* and *kdkA* from *H. influenzae* synthesized an LPS containing a single Kdo residue that was exclusively phosphorylated at position 4. The structure was determined by nuclear magnetic resonance spectroscopy of decacylated LPS. Therefore, the reaction products of both cloned Kdo kinases represent only one of the two chemical structures synthesized by *H. influenzae* I69.

**Haemophilus influenzae** is a nononenteric Gram-negative bacterium that is found in the human respiratory tract and may cause severe diseases, in particular septicemia and meningitis in children. One major virulence factor of this pathogen is the 3-deoxy-D-manno-oct-2ulosonic Acid (Kdo) Transferase (WaaA) and Kdo Kinase (Kdk) of *Haemophilus influenzae* Are Both Required to Complement a *waaA* Knockout Mutation of *Escherichia coli*.

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The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession numbers AJ277814, AJ277816, AJ277815, and AJ277817.

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The abbreviations used are: LPS, lipopolysaccharide(s); Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; mAb, monoclonal antibody; COSY, correlation spectroscopy; DQF, double quantum-filtered; ROE, rotating frame Overhauser effect; ROESY, rotating frame Overhauser effect spectroscopy; HMQC, heteronuclear multiple quantum coherence; HPACE, high performance anion exchange chromatography; PCR, polymerase chain reaction; bp, base pairs.
demonstrated together with an LPS-specific, ATP-dependent Kdo kinase from membrane extracts of *H. influenzae* (16). These data have been recently confirmed by the cloning and in vitro characterization of a Kdo kinase (kdkA) (17). However, due to limiting amounts of the in vitro reaction products of KdkA, the position to which phosphate had been transferred has not been determined.

We have established a cloning system based on a defined Re-type *E. coli* strain that is devoid of the host’s Kdo transferase activity and additionally harbors a ΔwaaCF mutation within the heptosyltransferase I and II genes involved in the consecutive transfer of glycerol-o-manno-heptose residues to Kdo. This strategy allowed us to characterize LPS that were synthesized in vitro by cloned Kdo transferases without interfering activity of the essential host-specific enzyme (18). Using this approach, we now show that the cloned monofunctional waaA from *H. influenzae* is not able to complement a knockout mutation within the corresponding gene of *E. coli*; however, cloning of both waaA and kdkA from a wild type or the 169 strain of *H. influenzae*, respectively, we are able to complement the mutation.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (4 × 10⁶ Bq/mmol) was obtained from ICN Biochemicals. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. CTP, NAD, isopropyl-β-D-thiogalactoside, hemin, and antibiotics were obtained from Sigma. Silica Gel 60 thin layer chromatography (TLC) plates were from Merck, Kdo and synthetic tetraacyl lipid A precursor compound 405 (1-monophosphoryl) and 406 (bisphosphoryl) (19) were gifts of P. Kosma (University of Vienna, Austria) and S. Kusumoto (Osaka University, Osaka, Japan), respectively. CMP-Kdo synthetase was partially purified from *Cytophaga* glutamicum R163/pJK2B14 as described (15), and lipid A 4'-kinase was prepared from *E. coli* BLR(DE3)/pJKB113 according to Garrett et al. (20).

**Plasmids, Bacterial Strain, and Growth Conditions**—Plasmids and bacterial strains used in this study are listed in Table I. *H. influenzae* strains were cultivated at 37 °C in brain-heart-infusion (Life Technologies, Inc.) supplemented with 10 mg/liter hemin and 10 mg/liter NAD as described (21). *E. coli* and *C. glutamicum* strains were cultivated at 37 and 30 °C, respectively, in Luria-Bertani medium (10 g/liter casein peptone, 5 g/liter yeast extract, 5 g/liter NaCl, pH 7.2; compounds were from Life Technologies) supplemented with the appropriate antibiotics (both strains: 20 mg/liter kanamycin sulfate; *E. coli* only: 50 mg/liter streptomycin sulfate, 12.5 mg/liter tetracycline/HCI, 100 mg/liter ampicillin). Isopropyl-β-D-thiogalactoside (1 mM) was added to induce recombinant genes that had been cloned under transcriptional control of the tac or trc promoter.

**General Cloning Techniques and Sequence Analysis**—Most DNA procedures were done according to standard techniques (22). All polymerase chain reactions (PCRs) used for cloning were performed with *Pfu* DNA polymerase (Stratagene), which exhibits proofreading activity. The digoxigenin-11-dUTP system (Roche Diagnostics) was used for DNA labeling and detection in Southern experiments according to the instructions of the manufacturer. DNA sequence analysis of both strands of the cloned genes was done by cycle sequencing with fluorescent dye terminators and sequence-specific primers on an ABI 377 sequencing automated (Perkin-Elmer). The computer programs gapped (IntelliGenetics), custalx1.8 (23), genedoc (K. B. Nicholas and H. B. Nicholas, Jr.), and blast (servers at the National Center for Biotechnology Information (NCBI)) were used to analyze DNA and deduced amino acid sequences.

**Plasmid Constructions**—The primers HindI primers (5'-ATATGGATCCGGAA-TTTCTTTAGTGGC-GGG-3', BamHI site underlined and start codon in boldface type) and HindII primers (5'-ATATGCACCGCGTCATTAGGGCTTCC-3', PstI site underlined and stop codon in boldface type) were used to amplify by PCR a 1299-bp waaA-encoding fragment from chromosomal DNA of *H. influenzae* 169. The amplificate was cut with BamHI and PstI and ligated with pCB20 (12), which had been linearized with the same restriction enzymes. The resultant construct, termed pCB20, encoded the Kdo transferase under transcriptional control of the tac promoter (Fig. 1A). The expression cassette with the tac promoter, waaA of *H. influenzae* 169, and the kanamycin resistance gene (kan) of pCB20 was amplified from pCB20 with the primers W151 (5'-TTTCTTTAGTGACCGTACCCCGG-3', SacI site underlined) and W152 (5'-AGAAGATTTCGCGCCACCGGGTGATG-3', SalI site underlined), cut with SacI, and ligated into the SalI site of pJSC2 (4). The construct with the orientation of the insert shown in Fig. 1B was selected and termed pJKB49. The recombinant strain was cultivated at 30 °C due to the temperature-sensitive origin of replication of pJSC2 (4). The kdkA gene was amplified by PCR from chromosomal DNA of *H. influenzae* DSM11121 and *H. influenzae* 169 using the primers HI3 (5'-ATATAC-
Kdo Transferase and Kdo Kinase from H. influenzae

ACGGTGATACCCCAATTATTTCACAG-3′, BspHI site underlined and start codon in boldface type) and HI4K (5′-TCATAATTAGTACCCCATATTGATAGAACTTGACGT-3′, KpnI site underlined and stop codon in boldface type). The 755-bp ampicillins were cut with BspHI and KpnI and ligated with pCP-TET (25), which had been linearized with EcoRI. The resultant plasmids were transformed into E. coli WBB34, respectively. The corresponding strains derived from pJKB113 and pJKB113A pose, the primers LACP (5′-CTCAGGTCACGGCTCTGG-3′) and HI4KAA2 (5′-ATTGACCCGATGTTTGAAG-3′) were used in the case of waaaA, and HIKDKA1 (5′-CTCTTGCGGTTCTTAATGCCG-3′) and HIKDKA2 (5′-TAAGGGATGACATGC-3′) were used in the case of kdkA.

Construction and Genetic Characterization of Chromosomal Knockout Mutations—The plasmids pJKB113 and pJKB113A were linearized with Pvull and ScaI and transformed into E. coli JCI7623 (26), which is recBC sbcBC and, thus, could be used to transfer the expression cassettes with the kdkA genes of the H. influenzae strains DSM11121 and H. influenzae 169 with additional sequences around the open reading frames were amplified from chromosomal DNA and were sequenced to determine the 5′- and 3′-ends of the genes including the binding sites of the cloning primers. For this purpose, the primer pairs HIWAAA1 (5′-GTTCCTGCAGTTAAATCC-3′) and HIWAAA2 (5′-ATCCGACGCTTTGGATTAGGC-3′) were used in the case of waaaA, and HIKDKK1 (5′-TTGCAGATTCCATGCG-3′) and HIKDKK2 (5′-TAAGGGATGACATGC-3′) were used in the case of kdkA.

Cloning and Sequencing of waaA from H. influenzae DSM11121—The waaA gene was amplified by PCR from chromosomal DNA of H. influenzae DSM11121 (identical to strain ATCC 51907, which was shown to be identical to the corresponding DNA region of H. influenzae strain JC7623 (26), which is recBC sbcBC) and the kanamycin resistance gene (9). The cells were centrifuged at 4 °C, washed 10 min with 200 ml of ice-cold 0.9% NaCl, 16 h with 200 ml of 96% ethanol, and twice for 3 h with 200 ml of acetone; and air-dried (yield: 1.1 mg of dry cells/liter). Bacterial LPS was obtained by extraction of the dried bacteria with phenol-chloroform-petroleum ether as described (32) (yield: 4.1%). Analyses of GlcN, Kdo, and phosphate were performed as described (29). LPS (50 mg, containing 30 μmol of GlcN) was de-O-acetylated by mild hydrazinolysis as described (33) (yield: 34 mg, 21 μmol of GlcN, 70%). De-O-acetylated LPS (30 mg) was subjected to de-N-acetylation by strong alkaline treatment as described (33). After neutralization with hydrochloric acid and desalting on Sephacryl G-10 (Amersham Pharmacica Biotech) in pyridinium acetate, pH 4.0, a single fraction was obtained, which was analyzed by analytical high-performance anion exchange chromatography (HPAEC) on a Dionex DX 500 chromatography system equipped with a Dionex CarboPac PA 1 column (4.5 × 500 mm), eluted at 1 ml/min using eluents A (water) and B (1 M sodium acetate, pH 6.0) and a linear gradient of 0–60% B in 70 min.

NMR Spectroscopy—NMR spectra were recorded on a solution of 5 mg of oligosaccharide (in 500 μl of D2O, 99.99%; Sigma) at pD 2.8 after pD 13. Hydrogen/deuterium interchanges by evaporation at reduced pressure. All spectra were recorded at a temperature of 300 K using standard Bruker DRX600 pulse programs. 1H NMR and 1H,13C COSY spectra were recorded on a Bruker DRX600 spectrometer (600.13 MHz, measured relative to acetone, 2,225 ppm) equipped with a 5-mm multinuclear inverse probe head with Z-gradient. One-dimensional 13C NMR (90.6 MHz, relative to acetone, 31.07 ppm) and 2D spectra (145.8 MHz, relative to 85% H3PO4, 77.0 MHz) were acquired on a Bruker DRX300 spectrometer equipped with a 5-mm multinuclear inverse Z-gradient probe head. 13C and 13C NMR chemical shift assignments were achieved by inverse heteronuclear multiple quantum coherence (HMQC) experiments (34) in phase-sensitive mode using states-time proportional phase incrementation (35). The 1H,13C HMQC spectrum was recorded by sampling 2048 data points in t2 and 256 increments of 32 scans in t1. Garp decoupling on proton was used during t2. The spectral width was 110 ppm in F1 and 10 ppm in F2. Prior to Fourier transformation, a qine window function was applied in F2, and a sine bell window function was applied in F1. The data matrix was zero-filled in F1 to 512 data points. The 1H,13C HMQC spectrum was recorded over a spectral width of 10 ppm in F2 and 14 ppm in F1. 256 increments of 40 scans each were collected acquiring 2048 data points in F2. 1H,13C COSY was performed in a phase-sensitive mode double quantum filtered (DQF-COSY) using the cosydqfpr program acquiring 4096 data points in F2 and 512 increments in F1 over a spectral width of 4496 Hz. Prior to Fourier transformation, the data matrix was multiplied with a squared sine bell window function. Coupling constants were determined on a first order basis. The data matrix was zero-filled in the F1 dimension to give a matrix of 4096 × 1024 data points. The ROESY experiment was performed using the roesypr pulse program with a mixing time of 250 ms collecting 2048 data points in the F2 dimension and 512 increments in the F1 dimension over a spectral width of 6009 Hz.

RESULTS

Cloning and Sequence Analysis of waaaA from H. influenzae 169—The waaaA gene was amplified by PCR from chromosomal DNA of H. influenzae 169, ligated downstream of the tac promoter into the E. coli plasmid shuttle vector pCB20 and transformed into E. coli XL-1Blue to give the plasmid pCB23 (Fig. 1A; for details, see Experimental Procedures). The nucleotide sequence of the cloned waaaA gene was determined and showed to be identical to the corresponding DNA region of H. influenzae DSM11121 (identical to strain ATCC 51907, which has been used to sequence the whole genome (36)).

In Vitro Characterization of the Cloned Kdo Transferase from H. influenzae 169—The plasmid pCB23 was transformed into the Gram-positive host C. glutamicum R163 from which cell
extracts were prepared and subjected to in vitro assays. The enzyme was able to transfer one Kdo residue to the acceptor 406 (Fig. 2, lane 3), and its activity depended on the presence of Kdo and CMP-Kdo synthetase (Fig. 2, lanes 3–6). CTP could be provided, but in limiting amounts, from cell extracts of recombinant C. glutamicum (Fig. 2, lane 7). A negative control with a cell extract from C. glutamicum R163/pCB20, which harbored the cloning vector without insert revealed no conversion of the (4'-32P)-radiolabeled compound 406 (data not shown). A small amount of an additional compound that had the same Rf value as the reaction product Kdo-406 formed by the recombinant WaaA of E. coli (cell extract from C. glutamicum R163/pJKB16) could be detected (Fig. 2, lanes 3 and 4). Therefore, Kdo-406 was isolated from a scaled up reaction mixture that had been performed with the cell extract of C. glutamicum R163/pCB23 (Fig. 3, lane 2), quantified, and used as an acceptor for the recombinant Kdo transferase of H. influenzae (Fig. 3, lanes 4–8). Almost no transfer of Kdo could be observed using Kdo-406 concentrations of 10 μM (Fig. 3, lane 4) or 100 μM (Fig. 3, lane 5). In the absence of a CMP-Kdo-generating system, even small amounts of 406 were liberated in an enzyme-dependent manner from Kdo-406 (Fig. 2B, lanes 6 and 8). A linear increase of Kdo-406 was observed within the first 20 min at 37 °C using acceptor 406 and the recombinant Kdo transferase from H. influenzae (Fig. 4, closed circle). The specific activity of the enzyme within the cell extract of C. glutamicum R163/pCB23 was calculated to 1.2 nmol min⁻¹ mg of protein⁻¹. Small amounts of Kdo-406 were also formed in vitro by the recombinant WaaA of E. coli (Fig. 4, open circle) under the same reaction conditions. However, Kdo-406 was identified as the major in vitro product of this enzyme (Fig. 4, open squares), which is known to be bifunctional (12). The specific activity of the Kdo-406 formation within the cell extract of C. glutamicum R163/pJKB16 was calculated to 6.7 nmol min⁻¹ mg of protein⁻¹. Thus, we concluded that the cloned waaA from H. influenzae encoded a monofunctional Kdo transferase, which was in contrast to the bifunctional activity of the corresponding enzyme from E. coli but in agreement with data published for membrane preparations from another strain of H. influenzae (16).

Cloning and Sequence Analysis of kdkA from H. influenzae DSM1121 and H. influenzae I69—The kdkA genes (17) encoding LPS-specific Kdo kinase were amplified from chromosomal DNA of H. influenzae DSM1121 and H. influenzae I69, and both DNA fragments were cloned into the plasmid pCF-TET (25) under transcriptional control of the tac promoter (Fig. 1C; for details, see “Experimental Procedures”). The corresponding derivatives of this plasmid with the kdkA genes were termed pJKB113 (H. influenzae DSM1121) and pJKB113A (H. influenzae I69). The nucleotide sequences of both kdkA genes were determined; that from H. influenzae DSM1121 was identical to the published one (36), whereas kdkA from H. influenzae I69 revealed 25 different nucleotides, six of which gave rise to altered amino
Inhibitory effects of Kdo2-406 on LPS from E. coli strains expressing Kdo transferase activity. The plasmid pJKB49 DNA was inserted into the single-knockout allele together with the waaA gene of H. influenzae—lacZ to form the plasmid pJKB16 (waaA from E. coli; lane 4). Complete reaction mixture with cell lysate from C. glutamicum R163/pKB16 (waaA from H. influenzae) and 10 μg Kdo-406; lane 5, as lane 4 but 100 μg Kdo-406; lane 6, as lane 5 but without Kdo; lane 7, as lane 5 but without CTP; lane 8, as lane 5 but without CMP-Kdo synthetase.

Characterization of LPS from Deep Rough E. coli Strains That Express kdkA and waaA of H. influenzae Using mAbs—LPS were analyzed in protein-free cell lysates of the bacteria by SDS polyacrylamide gel electrophoresis and silver staining (Fig. 5A). The LPS of all recombinant strains possessed similar electrophoretic mobilities as compared with the Re-type E. coli WBB01 (Fig. 5A, lane 2) or the deep rough H. influenzae strain (Fig. 5A, lane 3). A Western blot analysis with mAb A20 (31) (Fig. 5B), which recognizes a single Kdo residue, gave positive results with LPS of E. coli WBB01, WBB21, and WBB32 but not with the samples of H. influenzae I69 and E. coli WBB22 and WBB34. In contrast, LPS from the recombinant strains E. coli WBB22 and WBB34 reacted with mAb S42–16 (Fig. 5C), which recognizes α-Kdo-4-phosphate, but not with mAb S42–16 (Fig. 5D), which is specific for α-Kdo-5-phosphate (10). The specificity and sensitivity of mAb S42–16 was confirmed by the positive staining of LPS from H. influenzae I69 (Fig. 5D, lane 3).

Chemical Characterization of LPS from E. coli WBB22 and WBB34—LPS were extracted from E. coli WBB22 and WBB34 and subjected to successive de-O- and de-N-acylation. After gel permeation chromatography, one LPS fraction was obtained for both recombinant strains, which consisted of a single oligosaccharide eluting at 41.1 min in analytical HPAEC (Fig. 6, B and C). In contrast, LPS from H. influenzae I69 revealed two fractions by HPAEC, which eluted at 41.1 (I in Fig. 6A) and 46.2 min (II in Fig. 6A), respectively, and could be assigned by NMR analyses to α-Kdo-4P(2→6)-β-GlcN-4P(1→6)-α-GlcN-1P (I) and α-Kdo-4P(2→6)-β-GlcN-4P(1→6)-α-GlcN-1P (II).
Characterization of LPS from recombinant E. coli strains that express waaA and kdkA from H. influenzae DSM11121 and 169 using monoclonal antibodies. LPS from protein-free cell lysates of strain E. coli JC7823 (lane 1), E. coli WBB01 (lane 2), H. influenzae 169 (lane 3), E. coli WBB21 (lane 4), E. coli WBB32 (lane 5), E. coli WBB22 (lane 6), and E. coli WBB34 (lane 7) were separated by SDS polyacrylamide gel electrophoresis and stained with alkaline silver nitrate (not shown). The oligosaccharide obtained from the LPS of strain WBB32 was further characterized by nuclear magnetic resonance spectroscopy. 1H NMR spectra contained two signals of the same sugar sequence proved the substitution at position 6 observed between residues C (Kdo) and B. Comparison of chemical shift values with published data of substances that possess the same sugar sequence proved the substitution at position 6 of the β-GlcN residue (B), which is in accordance with all other bacterial lipopolysaccharides investigated so far. Since the results of Western blot and HPAEC analysis did not provide any evidence that the LPS of strain WBB34 differed from that of WBB22, the NMR analyses of the phosphorylated and deacylated carbohydrate backbone from LPS of strain WBB34 were not repeated.

In summary, E. coli WBB22 and WBB34 produced LPS composed of a single terminal α-Kdo-residue, which is exclusively phosphorylated at position 4 and is linked to the 6'-position of the lipid A backbone (β-GlcN-4P-(1→6)-α-GlcN-1P). It therefore represents only one of the two chemical structures known to be present in H. influenzae 169 LPS (Fig. 6A) (9, 10).

**DISCUSSION**

The waaA and kdkA genes of the Rd strain H. influenzae DSM11121 and the deep rough mutant 169 were cloned and sequenced. Both Kdo transferase genes had identical nucleotide sequences, whereas differences could be observed between the two Kdo kinases with respect to their gene and deduced protein sequences. A TBLASTN search with both KdkA amino acid sequences performed at NCBI and revealed positive results (E < 10^-18) with data from H. influenzae and the unfinished genomes of Acinetobacter actinomycetemcomitans, Pasteurella multocida, Vibrio cholerae, Shewanella putrefaciens and Bordetella pertussis. These bacteria belong to the families Pasteurellaceae or Vibrionaceae, members of which are known to possess LPS with single Kdo residues phosphorylated at position 4 (6). An amino acid sequence alignment of KdkA revealed a conserved amino acid sequence motif within the C-terminal half of all proteins, which closely resembles the consensus pattern of active sites from bacterial aminoglycoside phosphotransferases (38) as well as pro- and eukaryotic protein kinases (39) (Fig. 9A). The proposed consensus pattern for KdkA (Fig. 9B, I) only differs from that of serine/threonine-specific (Fig. 9B, 2; protost P500108) or tyrosine-specific (Fig. 9B, 3; protost P500109) protein kinases in a conserved arginine residue separated by one amino acid from the catalytically active aspartate. All different amino acids of the two proteins

**Fig. 5.** Characterization of LPS from recombinant E. coli strains that express waaA and kdkA from H. influenzae DSM11121 and 169 using monoclonal antibodies. LPS from protein-free cell lysates of strain E. coli JC7823 (lane 1), E. coli WBB01 (lane 2), H. influenzae 169 (lane 3), E. coli WBB21 (lane 4), E. coli WBB32 (lane 5), E. coli WBB22 (lane 6), and E. coli WBB34 (lane 7) were separated by SDS polyacrylamide gel electrophoresis and stained with alkaline silver nitrate (A) or blotted onto nitrocellulose membranes and developed with monoclonal antibody A20 (B; recognizing a single Kdo residue), S42 21 (C; recognizing α-Kdo-4P), or S42-18 (D; recognizing α-Kdo-5P).

**Fig. 6.** Analytical HPAE-chromatograms of deacylated LPS obtained from H. influenzae 169 (A), E. coli WBB22 (B), and E. coli WBB34 (C). I, α-Kdo-4P-(2→6)-β-GlcN-4P-(1→6)-α-GlcN-1P; II, α-Kdo-5P-(2→6)-β-GlcN-4P-(1→6)-α-GlcN-1P (data not shown). The oligosaccharide obtained from the LPS of strain WBB22 was further characterized by nuclear magnetic resonance spectroscopy. 1H NMR spectra contained two signals of anomeric protons at 5.722 and 4.854 ppm belonging to two GlcN residues (Fig. 7, A and B, respectively), as shown by full assignment of proton and carbon signals in two-dimensional 1H, 1H COSY and 1H, 13C HMOC spectra (Tables II and III). In addition, two signals of deoxy protons of a single α-Kdo residue (Fig. 7C) at 2.028 and 2.204 ppm (axial and equatorial H3) were present. The 31P NMR spectrum contained three signals at -1.5, 0.45, and 0.53 ppm. Glycosidic phosphorylation of residue A was evident from an additional coupling of its anomeric proton (J1,3P, 6 Hz), and couplings of its anomeric carbon (J1,3P, 5.2 Hz) and carbon C-2 (J1,3P, 8.8 Hz). Couplings of C-4 (J1,3P, 5.2 Hz) and C-5 (J1,3P, 7.0 Hz) of residue B and a downfield shift of carbon C-4 indicated phosphorylation at this position of residue B. Likewise, signals of carbons 4 (J1,3P, 4.6 Hz) and 5 (J1,3P, 3.0 Hz) of residue C (Kdo) were split due to phosphorylation at C-4, leading to far downfield shifts of signals of C-4 and in particularly of H-4 (Fig. 7). In DQF-COSY (Fig. 8), there was no signal of Kdo H-4 at higher field as would be expected for a Kdo residue phosphorylated in position 5. Therefore, position 4 was quantitatively phosphorylated, and phosphate at position 5 was not present. Sites of phosphorylation were confirmed by 1H,31P HMOC, which showed cross-correlation signals to protons at phosphorylated sites. These results thus confirmed the analytical HPAEC analysis of crude deacylated LPS, which showed a single peak corresponding to the 4'-phosphorylated molecule (Fig. 6). ROESY showed ROE contacts between H-1 of residue B and H-6a (weak) and H-6b (strong) of residue A. Thus, residue B was linked to position 6 of residue A. Since Kdo is a ketose lacking an anomeric proton, no ROE contacts were observed between residues C (Kdo) and B. Comparison of chemical shift values with published data of substances that possess the same sugar sequence proved the substitution at position 6 of the β-GlcN residue (B), which is in accordance with all other bacterial lipopolysaccharides investigated so far. Since the results of Western blot and HPAEC analysis did not provide any evidence that the LPS of strain WBB34 differed from that of WBB22, the NMR analyses of the phosphorylated and deacylated carbohydrate backbone from LPS of strain WBB34 was not repeated.

In summary, E. coli WBB22 and WBB34 produced LPS composed of a single terminal α-Kdo-residue, which is exclusively phosphorylated at position 4 and is linked to the 6'-position of the lipid A backbone (β-GlcN-4P-(1→6)-α-GlcN-1P). It therefore represents only one of the two chemical structures known to be present in H. influenzae 169 LPS (Fig. 6A) (9, 10).
from *H. influenzae* matched into nonconserved regions of all aligned sequences (data not shown), although two of them were located 13 residues upstream of the putative active site motif (Fig. 9A).

The recombinant *waaA* gene was expressed in *C. glutamicum*. This Gram-positive cloning host is devoid of LPS and Kdo, and thus, the activity of the cloned Kdo transferase could be studied without interference by host cell enzymes (13, 15). The cloned *WaaA* from *H. influenzae* was characterized in vitro as a monofunctional enzyme (Figs. 3 and 4), confirming the published data that have been obtained with the purified protein from a nontypeable *H. influenzae* strain (16). Differences in the activity of the enzyme to the bifunctional *WaaA* from *E. coli* (Fig. 4) were further evident from the observation that *waaA* from *H. influenzae* could not complement a knockout mutation within the corresponding gene of a deep rough *E. coli* strain. In contrast, this was possible by coexpressing *waaA* and *kdkA* from *H. influenzae*, and the resultant recombinant *E. coli* strains WBB22 and WBB34 displayed deep rough LPS containing a single phosphorylated Kdo residue (Figs. 5–8). Thus, the presence of at least two negatively charged groups within the inner core region seems to be a fundamental prerequisite for the functional integrity of the outer membrane in *E. coli*. This has been also suggested for the deep rough *H. influenzae* I69 (7–9). However, an isogenic mutant derived from a wild type *H. influenzae* has been constructed (40) and later was shown to harbor a *kdkA* knockout (17). This strain still allows the attachment of further core sugars and is viable under laboratory conditions but displays reduced virulence in vivo (40). Although detailed structural data on the LPS of this strain are lacking, a phosphorylated Kdo region seems not to be absolutely required for growth and multiplication of *H. influenzae*. Recently, Isobe

\begin{table}[h]
\centering
\caption{1H NMR (600.13 MHz) chemical shifts and coupling constants of the oligosaccharide obtained by successive de-O- and de-N-acylation of lipopolysaccharide of *E. coli* WBB22}

\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
Sugar & Chemical shift of proton (ppm) (coupling constants (Hz)) & 1 & 2 & 3ax & 3eq & 4 & 5 & 6a & 6b & 7 & 8a & 8b \\
\hline
Kdo4P & 2.028 (13, 13) & 2.204 (5) & 4.535 & 4.213 & 3.804 (11) & 3.948 & 3.906 (7, 13) & 3.653 (5) & 3.948 & 3.906 (7, 13) & 3.653 (5) \\
\hline
6αGlcN4P & 4.854 (8.6) & 3.127 (10) & 3.883 (7) & 3.923 & 3.746 & 3.773 (11) & 3.596 (7) & 3.948 (10) & 4.095 & 4.251 (3, 13) & 3.861 (8) \\
\hline
6βGlcN1P & 5.722* (3.3) & 3.421* (10) & 3.898 (10) & 3.596 (7) & 3.861 (8) & 3.653 (5) & 3.948 (10) & 4.095 & 4.251 (3, 13) & 3.861 (8) \\
\hline
\end{tabular}

\textsuperscript{a} J_{H,P}, 6 Hz.
\textsuperscript{b} J_{H,P}, 3.7 Hz.

\begin{table}[h]
\centering
\caption{13C NMR (90.25 MHz) chemical shifts and J C,P coupling constants of the oligosaccharide obtained by successive de-O- and de-N-acylation of lipopolysaccharide of *E. coli* WBB22}

\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|}
\hline
Sugar & Chemical shift of carbon atom (ppm) & 1 & 2 & 3 & 4 & 5 & 6a & 6b & 7 & 8 \\
\hline
αKdo4P & 172.6 & 99.3 & 33.2 & 70.8 & 65.4 & 72.0 & 69.2 & 63.2 \\
\hline
6αGlcN4P & 99.2 & 55.8 & 71.9 & 74.4 & 74.0 & 62.5 \\
\hline
6βGlcN1P & 92.0 & 54.2 & 69.6 & 69.7 & 72.8 & 69.2 \\
\hline
\end{tabular}

\textsuperscript{a} J_{C,P}, 4.6 Hz.
\textsuperscript{b} J_{C,P}, 3.0 Hz.
\textsuperscript{c} J_{C,P}, 5.2 Hz.
\textsuperscript{d} J_{C,P}, 7.0 Hz.
\textsuperscript{e} J_{C,P}, 5.2 Hz.
\textsuperscript{f} J_{C,P}, 8.8 Hz.

\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7}
\caption{1H NMR spectrum recorded at 600 MHz and chemical structure of deacylated LPS of *E. coli* WBB22. Assignments are shown for characteristic \textsuperscript{1}H reporter signals.}
\end{figure}

From *C. glutamicum* this Gram-positive cloning host is devoid of LPS and Kdo, and thus, the activity of the cloned Kdo transferase could be studied without interference by host cell enzymes (13, 15). The cloned *WaaA* from *H. influenzae* was characterized in vitro as a monofunctional enzyme (Figs. 3 and 4), confirming the published data that have been obtained with the purified protein from a nontypeable *H. influenzae* strain (16). Differences in the activity of the enzyme to the bifunctional *WaaA* from *E. coli* (Fig. 4) were further evident from the observation that *waaA* from *H. influenzae* could not complement a knockout mutation within the corresponding gene of a deep rough *E. coli* strain. In contrast, this was possible by coexpressing *waaA* and *kdkA* from *H. influenzae*, and the resultant recombinant *E. coli*
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