Inhibition of Lipopolysaccharide Biosynthesis and Cell Growth following Inactivation of the kdtA Gene in Escherichia coli*

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Charles J. Belunis§, Tony Clementz®, Sherry M. Carty**, and Christian R. H. Raetz‡§

From the Department of Biochemistry, Merck Research Laboratories, Rahway, New Jersey 07065 and the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

The enzyme 3-deoxy-D-manno-octulosonic acid (Kdo) transferase is encoded by the kdtA gene in Escherichia coli. The enzyme is a single polypeptide that catalyzes the transfer of two Kdo residues to a tetraacyldisaccharide-1,4-bisphosphate precursor of lipid A, designated lipid IVα (Belunis, C. J., and Raetz, C. R. H. (1992) Biol. Chem. 267, 9988–9997). To determine if Kdo transfer to lipid IVα is required for growth, we constructed a strain of E. coli with a chromosomal kdtA:kan insertion mutation. In mutants carrying the kdtA:kan allele on the chromosome, cell growth and Kdo transferase activity were dependent upon a copy of the intact kdtA gene on a plasmid. When the kdtA-bearing plasmid was itself temperature sensitive for replication, the growth of these strains was inhibited after several hours at 44 °C, and Kdo transferase activity in extracts became undetectable. Concomitantly, the cells accumulated massive amounts of lipid IVα, the precursor of (Kdo)2-lipid IVα. The kdtA:kan mutation could also be complemented by hybrid plasmids bearing the gseA gene of Chlamydia trachomatis. gseA specifies a distinct Kdo transferase that adds three Kdo moieties to lipid IVα. Lipopolysaccharide from E. coli kdtA:kan constructs complemented by gseA reacts strongly with antibodies directed against the genus-specific epitope of Chlamydia, whereas lipopolysaccharide from parental E. coli K-12 does not. Our studies prove that Kdo attachment during lipid A biosynthesis is essential for cell growth and accounts for the conditional lethality associated with mutations in Kdo biosynthesis.

The sugar 3-deoxy-D-manno-octulosonic acid (Kdo)3 appears to be an essential component of the lipopolysaccharide (LPS) of Escherichia coli and other Gram-negative bacteria (1–6). The enzyme Kdo transferase catalyzes the sequential addition of two Kdo sugars onto a molecule of lipid IVα (7, 8), a key precursor of lipid A (Fig. 1). Prior to its incorporation, Kdo is activated to CMP-Kdo by the enzyme CMP-Kdo synthase (9–11). Mutants of Salmonella typhimurium defective in Kdo biosynthesis are temperature sensitive for growth and accumulate several underacylated precursors of lipid A (12–14), the most abundant of which is lipid IVα (15, 16). Pharmacological inhibition of CMP-Kdo synthase in living cells has similar effects on LPS biogenesis (17–20).

Recently, Clementz and Raetz (21) described a colony screening technique to identify E. coli mutants with thermolabile Kdo transferase in cell extracts. Using these mutants, they were able to isolate the Kdo transferase gene, designated as kdtA (21). Although cell extracts of these mutants contain <5% of wild-type Kdo transferase activity, when assayed at 42 °C, the strains do not display temperature-sensitive growth, and they do not accumulate lipid IVα at 42 °C (21). It appears that these mutants retain enough residual Kdo transferase activity in vivo to synthesize adequate levels of (Kdo)2-lipid IVα.

Kdo is a component of capsular polysaccharides in some strains of E. coli and related bacteria (6, 22, 23). Since Kdo-containing polymers other than LPS are not found in all Gram-negative bacteria (6, 22, 23), it seems unlikely that the presence of Kdo in these polymers would account for the conditional lethality of the Kdo biosynthesis mutants. Nevertheless, the question of whether or not Kdo transfer to lipid IVα per se is essential for cell growth could only be tested by examining the consequences of complete inactivation of the kdtA gene.

In this paper, we demonstrate that the kdtA gene is indeed essential for growth of E. coli by constructing a kdtA::kan insertion mutation, using a gene replacement method (24). Growth of this strain is absolutely dependent upon the presence of a functional copy of the kdtA gene (or the related gseA gene) carried on a plasmid.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP, [α-2P]dCTP, and 25P, were obtained from Amersham International. CTP and Kdo were obtained from Sigma. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim. Other items were purchased from the following companies: yeast extract and tryptone (Difco Laboratories, Detroit, MI); Silica Gel 60 thin layer plates, 0.25 mm (E. Merck, Darmstadt, Germany); PRIME IT random primer kit (Stratagene); and Hybond nylon membranes (Amersham Corp.). The primary mouse monoclonal antibody CT403.1 (Becton-Dickinson, Research Triangle Park, NC). The secondary antibody (horseradish peroxidase-conjugated goat anti-mouse) was from Promega. Lipid IVα and 4-2P lipid IVα were prepared as described (7, 15). CMP-Kdo synthase was partially purified from E. coli (7).

Plasmids, Bacterial Strains, and Growth Conditions—All plasmids and bacterial strains employed in this study are listed in Table I. The medium for growth of cells in liquid culture or on agar plates was Luria broth (LB), consisting of 5 g of yeast extract, 10 g of tryptone, and 10 g of NaCl per liter (25). Media were supplemented with ampicillin (125 µg/ml), tetracycline (25 µg/ml), chloramphenicol (10 µg/ml), or kanamycin (20 µg/ml) as indicated to select for cells resistant to these drugs. Sensitivity or resistance to UV light was determined by survival after irradiation with 254 nm UV light as described by Ausubel et al. (26).

DNA Techniques—E. coli chromosomal DNA was isolated as described by Ausubel et al. (26). Large-scale preparation of plasmid DNA was carried out by the method of Birnboim and Doly (27). Specific DNA

**Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710. To whom correspondence should be addressed.

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§ Present address: Dept. of Inflammation and Autoimmune Diseases, Hoffmann-La Roche, 340 Kingsland St., Nutley, NJ 07110.

† Supported by a National Institutes of Health training grant in biological chemistry to Duke University.

** Present address: Dept. of Biochemistry, Duke University Medical Center, Durham, NC 27710. To whom correspondence should be addressed.

The abbreviations used are: Kdo, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharide; kb, kilobase(s).
The six enzymatic reactions leading to the formation of lipid IVₐ include the transfer of Kdo to the 8 position of the outer Kdo sugar. The kdtA gene is involved in this process. kdtA is a bifunctional enzyme catalyzing both the first and the second Kdo transfers, as indicated (8, 21). The Kdo transferase encoded by the gseA gene of C. trachomatis (not shown) catalyzes an additional, third Kdo transfer to the 8 position of the outer Kdo sugar (33).

**Fig. 1.** Kdo transfer and the completion of lipid A assembly in E. coli. The six enzymatic reactions leading to the formation of lipid IVₐ probes were labeled with [α-32P]dCTP using the PRIME IT random primer kit (Stratagene) according to manufacturer’s specifications. Southern blot hybridizations were performed as described by Maniatis et al. (29). Restriction endonucleases and T4 DNA ligase were used according to the manufacturer’s specifications. DNA fragments were isolated from agarose gels using Geneclean (Bio-Sp Bio-Spin). All other techniques were adapted from Ausubel et al. (26).

Transformation of E. coli Cells—E. coli cells were made competent for transformation by CaCl₂ treatment as described previously (30). Transformants were plated on LB containing the required antibiotic and incubated at 30 or 37°C as indicated.

Kdo Transferase Assay—Kdo transferase activity was assayed as described previously by Brozek et al. (7). Reaction mixtures contained 50 mM Hepes, pH 7.0, 10 mM MgCl₂, 3.2 mM Triton X-100, 5 mM CTP, 2 mM Kdo, 100 μM 4-32P-labeled lipid IVₐ (3–6 × 10⁸ cpm/nmol), 1.8 milliunits of CMP-Kdo synthase, and enzyme protein in a total volume of 20 μl. Assays were carried out for 10–20 min at 30°C in 0.6-ml Eppendorf tubes. Reactions were terminated by spotting 5 μl of the assay mixtures onto silica thin layer plates. The plates were air dried and then developed in chloroform/methanol/formic acid/water (30:70:16:10, v/v). Alternatively [1-14C]Kdo (3200 cpm/nmol) was used as the labeled substrate (21). The 32P- or 14C-labeled products were located by autoradiography, scraped into scintillation vials, and counted in 10 ml of Biosafe mixture (Research Products Intl., Mount Prospect, IL). In some experiments, the labeled product was detected with an automatic TLC linear analyzer (Berthold Analytical Instruments, Nashua, NH). A unit of enzymatic activity is defined as the amount of enzyme required to catalyze the formation of 1 nmol of product per min. Specific activity is defined as units per mg of protein (8).

Plasmid Construction—To construct pJSC20 (Fig. 2), pMAK705 (24) and pCL3 (21) were digested with BamHI and HindIII. The 3-kb fragment from pCL3 containing kdtA, was purified by gel electrophoresis and ligated into pMAK705. The ligation mixture was used to transform competent cells of J M109 (31). Plasmid-bearing cells were selected by growth on LB plates containing chloramphenicol at 30°C. Surviving colonies were tested for the presence of the desired insert, and extracts were assayed for elevated levels of Kdo transferase activity. pJSC20 contained the predicted insert with its characteristic, single Sall restriction site located within the kdtA gene (21).

pJSC2 and pUC-4K were digested with Sall. The kanamycin cassette from pUC-4K was purified by gel electrophoresis and ligated into pJSC2. The ligation mixture was used to transform competent cells of J M109. Plasmid-bearing cells were selected by growth at 30°C on LB plates containing chloramphenicol and kanamycin. pJSC20 showed the expected restriction sites, and extracts did not express elevated levels of Kdo transferase activity, indicating disruption of the plasmid-born kdtA gene. Cells carrying pJSC20 grew well in the presence of chloramphenicol and kanamycin at 30°C but not at 44°C, confirming that the plasmid pJSC20 is temperature sensitive for replication.

Construction of a Mutant with an Insertion in the Chromosomal Copy of kdtA—The construction of CJB26 (Table I) was carried out using the methods of Hamilton et al. (24). Competent cells of MC1061 (32) were transformed with pJSC20. Following growth to mid-log phase at 30°C, cells were plated on prewarmed LB plates, containing chloramphenicol and kanamycin, and incubated at 44°C. Cells in which the plasmid had integrated into the chromosome were selected by growth at 44°C. A single colony of such a cotransformant was used to inoculate 1 ml of LB broth, containing chloramphenicol, and was incubated at 30°C for 6 h. Next, the culture was used to inoculate 100 ml of LB broth, containing chloramphenicol, and was grown to stationary phase at 30°C. A portion of the culture was then diluted 1:100,000, and the cells were grown to stationary phase. This process was repeated a second time, given that at 30°C the cotransformants are unstable and can excise (Fig. 3) to generate a free plasmid, carrying either the wild-type kdtA gene or the kdtA-kan allele (24). Following the above cycles of outgrowth, the cells were plated on LB medium containing chloramphenicol at 30°C. Plasmid-containing cells were identified by their inability to grow to 44°C on chloramphenicol plates. Rapid plasmid screens were performed on 15 of such temperature-sensitive strains. Plasmids isolated from 6 of these strains were the same size as pJSC20. The other 9 strains contained smaller plasmids, indicating that the kdtA::kan insertion of pJSC20 have been reviewed elsewhere (2). The chromosomal kdtA gene of E. coli is a bifunctional enzyme catalyzing both the first and the second Kdo transfers, as indicated (8, 21). The Kdo transferase encoded by the gseA gene of C. trachomatis (not shown) catalyzes an additional, third Kdo transfer to the 8 position of the outer Kdo sugar (33).
had replaced the wild-type kdtA gene on the chromosome (Fig. 3). One of these isolates (designated CJB26) was made recA<sup>2</sup> by P1 transduction using JC10241 (32) as the donor. The presence of the recA<sup>2</sup> phenotype in CJB26 was confirmed by its sensitivity to UV light.

Strain NEB1 was constructed by transforming competent cells of CJB26 with pKEM1, which contains the C. trachomatis gseA gene (33). The kdtA<sup>-</sup>-bearing plasmid of CJB26 was cured by growth at 44°C in the absence of chloramphenicol but in the presence of ampicillin to select for pKEM1. The presence of pKEM1 as the only plasmid in the surviving colonies was confirmed by rapid plasmid isolation and restriction digestion analysis.

Phospholipid Analysis—Phospholipids were labeled, extracted, and analyzed as described (34, 35). Steady-state phospholipid composition was determined by uniformly labeling cells in LB medium containing <sup>32</sup>P (20 μCi/ml). Fresh LB containing <sup>32</sup>P (20 μCi/ml) was used to dilute cultures when necessary. Radiolabeled phospholipids were extracted from cells by transferring 0.8 ml of the cultures to glass tubes containing 3 ml of chloroform/methanol (1:2 v/v). The contents of the tubes were mixed and allowed to stand at room temperature for 1 h. The tubes were centrifuged for 20 min at low speed to remove insoluble material. The supernatants were transferred to new glass tubes containing 10 μg of E. coli phospholipids. A two-phase system was made by the addition of 1 ml of chloroform and 1 ml of 0.2 M HCl. The contents of the tubes were mixed, and the resulting phases were separated by a brief centrifugation. The upper phases were removed, and each lower phase was concentrated by evaporation under a stream of nitrogen. A portion of the phospholipids (5 × 10<sup>4</sup> cpm) was spotted onto silica gel plates. The phospholipids were separated by thin layer chromatography in the solvent chloroform/pyridine/88% formic acid/water (40:60:16:5 v/v). The <sup>32</sup>P-labeled phospholipids were located by autoradiography.

**RESULTS**

**Temperature-sensitive Growth of Strain CJB26—** E. coli strain CJB26 was derived by replacement of the chromosomal copy of kdtA with the kan element disrupted kdtA gene of pJSC20 by homologous recombination (Fig. 3, reaction B). The recA<sup>−</sup> gene was subsequently introduced by P1 transduction.

### Table I

| Strain/plasmid | Relevant genotype | Source or reference |
|---------------|------------------|---------------------|
| E. coli J M109 | recA<sup>−</sup> Δlac-pro endA gyrA96 thi-1 | Pharmacia |
| MC1061        | araD139 Δ(ara-leu) 7697 hsdM<sup>+</sup> | Crowell et al. (32) |
| J SC20        | kdtA::kan (derivative of MC1061)/pJSC20 | This work |
| CJ B26        | kdtA::kan recA<sup>−</sup> (derivative of J SC20 by P1 transduction)/pJSC20 | This work |
| NEB1          | kdtA::kan recA<sup>−</sup> (derivative of CJ B26 containing plasmid pKEM1) | This work |
| S. typhimurium ST150 | kdsA (temperature sensitive) | M. J. Osborn (15) |
| Plasmids      |                  |                     |
| pCL3          | kdtA<sup>−</sup> amp | Clementz and Raetz (21) |
| pMAK705       | cam (temperature sensitive for replication) | Hamilton et al. (24) |
| pUC-4K        | kan amp | Pharmacia |
| pJSC20        | kdtA<sup>−</sup> (derivative of pMAK705) | This work |
| pJSC2         | kdtA::kan (derived from pJSC2) | This work |
| pKEM1         | gseA<sup>−</sup> (derived from pUC18) | F. Nano (33) |

**Fig. 2.** pJ SC20 contains a kdtA gene disrupted by a kan insertion. The construction of pJ SC20 is described in detail under “Experimental Procedures.” The selections for plasmids pJ SC2 and pJ SC20 were based on chloramphenicol resistance or chloramphenicol and kanamycin resistance, respectively. The chloramphenicol resistance gene and the temperature-sensitive replicon are indicated as cam and rep, respectively.

**Fig. 3.** Construction of an E. coli strain bearing a chromosomal kdtA gene disrupted by a kan insertion. The construction of CJ B26 (Table I), which is based on homologous recombination at 44°C followed by resolution of the cointegrate at 30°C, is described in detail under “Experimental Procedures.” Pathway B, leading to CJ B26, leaves the inactivated kdtA gene behind on the chromosome. The covering plasmid of CJ B26 is identical to pJ SC2, as drawn in Fig. 2. The chloramphenicol resistance gene and the temperature-sensitive replicon are indicated as cam and rep, respectively.
using JC10241 (32) as the donor. CJ B26 is chloramphenicol and kanamycin resistant at 30 °C. The excised, recombinant plasmid in strain CJ B26 (Fig. 3) is identical to pJSC2 (Fig. 2) and is temperature sensitive for replication. Shifting the growth temperature from 30 to 44 °C inhibits replication of the plasmid DNA and the transfer of the intact kdtA gene (and chloramphenicol resistance) to daughter cells.

To determine whether or not the kdtA gene is essential for growth, cells of CJ B26 from an overnight culture grown at 30 °C in the presence of chloramphenicol were inoculated into LB medium lacking chloramphenicol at A600 of 0.1 (Fig. 4). Cells were then cultured at 44 °C with intermittent back dilution to maintain the A600 between 0.06 and 0.6. The results are plotted as a cumulative growth yield at 44 °C (Fig. 4). After about 4 h, the plating efficiency of CJ B26, as judged by growth of single colonies on LB agar lacking chloramphenicol at 30 °C (Fig. 4), stopped increasing. However, the A600 of CJ B26 continued to rise slowly. The control strain, MC1061/pJSC2, continued to grow rapidly after the temperature shift, since the plasmid copies of the kdtA gene were not needed in MC1061 for growth in LB medium at 44 °C without chloramphenicol (Fig. 4). The results of Fig. 4 demonstrate that the kdtA gene is essential.

Loss of Kdo Transferase Activity at 44 °C—Kdo transferase activity was assayed in extracts of both MC1061/pJSC2 and CJ B26 following a temperature shift from 30 to 44 °C (Fig. 5). In LB medium lacking chloramphenicol. The specific activity of Kdo transferase in extracts of MC1061/pJSC2 was ~3-fold higher than wild type at the time of temperature shift but gradually declined to wild-type levels after 2.5 h at 44 °C (Fig. 5). This behavior is consistent with the loss of pJSC2. The specific activity of Kdo transferase in extracts of CJ B26 at the time of the temperature shift was approximately 2 nmol/min/mg lower than MC1061/pJSC2 (Fig. 5), consistent with the lack of the chromosomal copy of kdtA in CJ B26. The specific activity in extracts of CJ B26 decreased to wild type levels after 2 h at 44 °C (Fig. 5) but continued to drop until no activity was detected in extracts from cells that had been held at 44 °C for 7 h. The time at which the Kdo transferase specific activity in extracts of CJ B26 dropped to levels below wild type (Fig. 5) was about the same time at which cell viability stopped increasing (Fig. 4).

Effect of Plasmid Loss on Lipid IVa Accumulation in CJ B26—Temperature-sensitive S. typhimurium mutants defective in Kdo biosynthesis accumulate underacylated lipid A disaccharide precursors when grown at 42 °C (12, 13). For instance, when the kdsA-deficient mutant STi50 is uniformly labeled with 32P at 30 °C and then shifted to 42 °C, one observes massive accumulation of lipid A precursors, as judged by thin layer chromatography (15, 16). The predominant precursor is lipid IVa (Fig. 1), which represents as much as 5–10% of the chloroform-soluble substances extracted from the bacteria under non-permissive conditions. In wild-type cells, lipid IVa represents less than 0.1% of the total chloroform-soluble polar lipids.

In the experiment of Fig. 6, cells of mutant STi50 and CJ B26 were grown in parallel at 30 °C and were uniformly labeled with 32P. The STi50 culture was shifted to 44 °C when the A600 reached 0.8. A portion was harvested just before and 2 h after the temperature shift for analysis of lipid composition. The CJ B26 culture was first diluted to an A600 of 0.1 with excess medium containing 32P, and then it was shifted to 44 °C. The CJ B26 culture was given time to lose its covering plasmid and Kdo transferase activity by intermittent 10-fold back dilution in media containing 32P, whenever the A600 had reached 0.6. Over the course of 9.5 h, portions of the CJ B26 culture were removed, and the phospholipids were extracted under acidic Bligh-Dyer conditions (34). Samples of ~5 × 104 cpm of uniformly labeled phospholipids obtained at each time point were spotted onto a silica gel plate, which was developed in chloroform/pyridine/88% formic acid/water (40:60:16.5:5 v/v/v/v). The plate was analyzed by autoradiography (Fig. 6). Cells of the kdsA-deficient mutant STi50 accumulated large amounts of lipid IVa after 2 h at 44 °C (Fig. 6). Prior to the temperature shift, the lipids of CJ B26 consisted mainly of glycosylphospholipids, which migrated rapidly in the solvent system employed.
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Fig. 6. Lipid IVₐ accumulation in CJB26 grown at 44 °C and in a temperature-sensitive mutant of S. typhimurium defective in Kdo biosynthesis. Cells of the S. typhimurium mutant ST150 and the E. coli strain CJ B26 were grown for three generations at 30 °C on LB broth containing ³²P (20 μCi/ml). When the absorbance at 600 nm had reached 0.8, the ST150 cells were shifted to 44 °C, and the CJ B26 culture was diluted to an A₆₀₀ of 0.1 prior to the temperature shift. To maintain exponential growth, the CJ B26 culture was diluted 1:10 whenever the A₆₀₀ reached 0.6. At the time of the temperature shift (designated 0) and at the indicated times, samples were withdrawn, and the lipids were extracted under acidic conditions (34). A portion of the radioactive lipids was spotted onto a silica gel plate that was developed in chloroform/pyridine/88% formic acid/water (40:60:16:5 (v/v)). The glycerophospholipids migrated rapidly, whereas lipid IVA migrated more slowly. The accumulation of lipid IVA is detectable in ST150 after 2 h at 44 °C, and in CJ B26, after 6 h at 44 °C. Lipid IVₐ is not detectable in ST150 at 30 °C or CJ B26 at 30 °C. Wild-type E. coli cells also do not accumulate lipid IVₐ at 30 °C or during prolonged incubations at 44 °C (data not shown).

After 6.5 h at 44 °C, CJ B26 also accumulated lipid IVₐ (Fig. 6). The timing of lipid IVₐ accumulation approximately coincided with the complete loss of measurable Kdo transferase activity (compare Figs. 4 and 6). Wild-type E. coli do not accumulate lipid IVₐ at any growth temperature.

Rescue of the kdtA::kan Mutation of CJ B26 by the C. trachomatis gseA Gene—Strain NEB1 (Table I) was constructed by transforming CJ B26 with pKEM1, followed by selection for growth at 44 °C to dilute out plasmid. NEB1 was sensitive to UV light, but it was ampicillin and kanamycin resistant at both 30 and 40 °C. NEB1 grew more slowly than did MC1061 (Fig. 7), especially at 42°C in shaking culture. NEB1 was nevertheless able to form colonies on LB agar at 44–46 °C.

Southern blots of chromosomal DNA isolated from MC1061 and NEB1 were prepared and probed with a ³²P-labeled 1.7-kb EcoRV fragment containing the complete kdtA gene. Restriction digestion of the chromosomal DNA with EcoRV should produce fragments of 1.7 kb from cells containing the wild-type kdtA gene (MC1061) and 3 kb from cells containing the kan insertion within the kdtA gene (NEB1). Digestion of DNA isolated from NEB1 with Clal should produce fragments of approximately 1.8 and 1.2 kb because of a single Clal site inside the kan gene. The predicted fragments were indeed observed (Fig. 8). These results confirmed that the kan cassette was located within the kdtA gene of NEB1 and, by inference, of CJ B26.

Extracts of the kdtA point mutant TC5 (21) contain <5% of wild-type Kdo transferase activity when assayed at 42 °C. Extracts of TC5 cells transformed with the gseA-bearing plasmid pKEM1 regain Kdo transferase activity at 42 °C, generating not only Kdo₂-lipid IVₐ, but also Kdo₃-lipid IVₐ (33). Extracts of NEB1 were similarly capable of transferring three Kdos onto lipid IVₐ (Fig. 9, panel A) in the absence of any residual KdtA activity. The steady state level of Kdo₂-lipid IVₐ (Fig. 9, panel A) is somewhat higher in the reaction catalyzed by the gseA gene product than by the kdtA-encoded enzyme (Fig. 9, panel B). This observation suggests that the two Kdo transferases differ not only with respect to their ability to incorporate the third Kdo residue but also in the relative rates at which they catalyze the first and second glycosylations.

Specific Binding of an Antibody Directed Against the Genus-specific Epitope by NEB1—As shown in the dot blots of Fig. 10, unfractoned cell extracts of NEB1 react strongly with a mouse monodonal antibody (CT403.1) directed against the genus-specific epitope of C. trachomatis, whereas comparable preparations of MC1061 do not. Even at 10-fold higher levels of primary antibody or cell extracts (not shown), no genus-specific epitope is detected in MC1061.

The phospholipid compositions of NEB1 and MC1061, grown in LB broth at 37 °C, were analyzed by ³²P labeling, as in Fig. 6. No accumulation of lipid IVₐ was observed in NEB1 (data not shown), demonstrating that GseA can function in vivo at a rate that is comparable to KdtA. The slow growth of NEB1 (Fig. 7) therefore cannot be attributed to inefficient Kdo transfer and lipid IVₐ accumulation.

DISCUSSION

Previous genetic and pharmacological studies have demonstrated that the biosynthesis and activation of Kdo are essential processes in Gram-negative bacteria (12–20). When the formation of CMP-Kdo is blocked, cells accumulate large amounts of lipid IVₐ, consistent with the pathway shown in Fig. 1 (12–20). However, given that Kdo can be a constituent of other cell surface molecules (6, 22, 23), it is unclear whether the attachment of Kdo to lipid IVₐ per cell is required for growth. An E. coli gene encoding a bifunctional Kdo transferase (kdtA) has previously been identified and sequenced (21), but no conditional alleles or insertion mutations of kdtA have been reported. The physiological consequences of selective inhibition of Kdo transfer to lipid IVₐ therefore remain uncertain.

Using homologous recombination, we have now constructed an E. coli strain (CJ B26) with a kan element insertion in the chromosomal copy of the kdtA gene (Fig. 3, pathway B). CJ B26 retains a functional copy of kdtA on a hybrid plasmid (Fig. 3, pathway B) that is equivalent to pJSC2 (compare Figs. 2 and 3). Since pJSC2 is derived from pMAK705 (24) and harbors a temperature-sensitive pSC101 replicon (Fig. 2), pJSC2 is capable of replicating at 30 °C but not at 44 °C. Cells of CJ B26 are expected to survive only at the lower temperature if the kdtA gene is essential but would grow at both temperatures if the kdtA gene is not essential.

As shown by the behavior of CJ B26 in the experiments of Figs. 4–6, the kdtA gene is indeed essential. Cells of CJ B26 grown at 44 °C lose the ability to synthesize Kdo transferase as the plasmid is cured (Fig. 5). The decrease in Kdo transferase activity with time precedes the accumulation of lipid IVₐ in the cells (Fig. 6). As Kdo transferase activity falls below wild-type levels, growth slows and eventually ceases (Fig. 4). Only those daughter cells that retain the functional copy of kdtA on a plasmid can grow, while the other daughter cells cannot. Viable cell counts on LB plates both with and without chloramphenicol supplementation at 30 °C are identical in cultures of CJ B26 shifted to 44 °C (data not shown). The absence of measurable Kdo transferase in extracts of CJ B26 after 6 h at 44 °C (Fig. 5) indicates that there are no additional genes encoding for Kdo transferase isoenzymes in E. coli.

The decrease in the growth rate of CJ B26 and the accumu-
lation of lipid IV\textsubscript{A} during plasmid curing at 44 °C are similar to what occurs when temperature-sensitive Kdo biosynthesis mutants of S. typhimurium (12–16) are shifted to non-permissive conditions or when CMP-Kdo synthase inhibitors are added to wild-type Gram-negative bacteria (17–20). Our findings show that inhibition of LPS biosynthesis is sufficient to explain the antibacterial effects of CMP-Kdo synthase inhibitors and the conditional lethality of mutations in Kdo biosynthesis. It is still uncertain whether Kdo\textsubscript{2}-lipid IV\textsubscript{A} is actually required for growth or whether the accumulation of lipid IV\textsubscript{A} is toxic.

In previous studies (33), we showed that the gseA gene of C. trachomatis codes for a novel Kdo transferase. The C. trachomatis Kdo transferase can add at least one additional Kdo onto Kdo\textsubscript{1}-lipid IV\textsubscript{A}. Preliminary results also indicated that GseA might actually recognize lipid IV\textsubscript{A} as a substrate (33), suggesting that GseA is a trifunctional Kdo transferase. The fact that we were able to replace the temperature-sensitive covering plasmid (pJSC2) present in CJB26 with a thermostable plasmid (pKEM1) bearing only gseA (33) provides strong support for the ability of GseA to use lipid IV\textsubscript{A} as a substrate in living cells. As expected from the ability of pKEM1 to rescue CJB26 at 44 °C, extracts of NEB1 were able to catalyze the formation of Kdo\textsubscript{1}-, Kdo\textsubscript{2}-, and Kdo\textsubscript{3}-lipid IV\textsubscript{A} from lipid IV\textsubscript{A} (Fig. 9), despite

![Fig. 7. Growth of MC1061 and NEB1 at various temperatures.](image)

![Fig. 8. Southern blot of chromosomal DNA from NEB1 and MC1061.](image)

![Fig. 9. Kdo transferase activity in extracts of NEB1 and MC1061.](image)
Inactivation of the kdtA Gene

FIG. 10. Dot blot analysis of extracts of NEB1 and MC1061 using a monoclonal antibody directed against the genus-specific epitope of C. trachomatis. Cells of MC1061 and NEB1 were grown to late log phase in LB broth. A portion of each culture (1 ml) was centrifuged to recover the cells. The pellets were resuspended in 200 μl of 0.1% NaCl loading buffer (50 μl Tris-chloride, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 700 μl 2-mercaptoethanol), and the lysed samples were diluted with phosphate-buffered saline to final protein concentrations of 3–15 μg/ml. Next, 1-μl portions containing the amount of protein indicated were spotted onto a nitrocellulose membrane (0.45 micron Hybond-C super from Amersham) and allowed to dry for 5 min at 42°C. The membrane was blocked for 1 h at room temperature in 30 ml of phosphate-buffered saline containing 6% K Roger nonfat dry milk and 0.02% sodium azide. The primary antibody (a 1 mg/ml stock of CT403.1) was exposed to the membrane at a 1:5000-fold dilution in a fresh 30-ml portion of the above blocking buffer for 1 h. The membrane was rinsed three times with phosphate-buffered saline. Next, it was incubated for 1 h in another 30-ml portion of the above blocking buffer supplemented with secondary antibody at a 1:1000 dilution from a 1 mg/ml stock of Promega horseradish peroxidase conjugated goat anti-mouse antibody. After several final washes with phosphate-buffered saline and distilled water, the genus-specific epitope was detected on the membrane by a 1-min incubation in 40 ml of enhanced chemiluminescence reagents (ECL Western blotting detection reagents from Amersham), followed by a 5-min exposure to Kodak X-Omat X-ray film.

The insessional inactivation of the kdtA gene (Fig. 8). Why NEB1 cells grow more slowly than wild type, especially at elevated temperatures (Fig. 7), is uncertain, but it is not the result of lipid IVα accumulation.

Southern blot analysis (Fig. 8) confirmed the presence of the kdtA gene on a chromosomal DNA for Southern blotting, since the recombinant covering plasmid in NEB1 did not contain any kdtA sequences. A hybridizing band on a Southern blot could only be due to kdtA sequences present on the chromosome and not from contaminating plasmid DNA. Because of the latter issue, we did not use CJ B26 grown at 44°C as the source of genomic DNA.

In summary, we have constructed a strain of E. coli with an insertion mutation in the kdtA gene. Biosynthesis of Kdo2-lipid IVα and growth of the organism are absolutely dependent on the presence of a functional copy of an intact kdtA gene on a plasmid. The mutation can also be complemented by the gseA gene from C. trachomatis. The strain NEB1, which contains the kdtA::kan mutation on the chromosome and gseA on a plasmid, is viable, and it can synthesize Kdo-containing LPS that is recognized by an antibody directed against the genus-specific epitope (Fig. 10). It will be interesting to determine the precise structure of the LPS made by NEB1. Since NEB1 appears to make Kdo2-containing LPS (Fig. 10), the strain could prove to be a valuable source of the epitope for diagnostic assays and structural studies. NEB1 should also prove useful for the isolation of the trifunctional Kdo transferase, since no bifunctional Kdo transferase is present to complicate enzymatic assays.

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Charles J. Belunis, Tony Clementz, Sherry M. Carty and Christian R. H. Raetz

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