A Mutant of Escherichia coli Defective in the First Step of Endotoxin Biosynthesis*

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Using localized mutagenesis of whole cells, we have isolated a temperature-sensitive UDP-N-acetylglucosamine acyltransferase mutant of Escherichia coli that loses all detectable acyltransferase activity and quickly dies after a shift from 30 to 42 °C. Acyltransferase activity and temperature resistance are restored by transforming the mutant with a hybrid plasmid containing the E. coli gene for UDP-GlcNAc acyltransferase (lpxA). In addition, a new assay has been developed for quantitating the amount of lipid A (the active component of endotoxin) in E. coli and related Gram-negative strains. Cells are labeled with 32P, and extracted with chloroform/methanol/water (1:2:0.8, v/v) to remove glycerophospholipids. The residue is then hydrolyzed with 0.2 M HCl to liberate the “monophosphoryl” lipid A degradation products (Qureshi, N., Cotter, R. J., and Takayama, K. (1986) J. Microbiol. Methods 5, 65–77), each of which bears a single phosphate residue at position 4'. The amount of lipid A is normalized to the total amount of labeled glycerophospholipid present in the cells. The steady state ratio of lipid A to glycerophospholipid in wild-type cells is approximately 0.12. The lipid A content of the acyltransferase mutant is reduced 2-3-fold, and the rate of lipid A synthesis is reduced 10-fold when compared to wild-type after 60 min at 42 °C. These results provide physiological evidence that UDP-N-acetylgalactosamine acyltransferase is the major committed step for lipid A biosynthesis in E. coli and that lipid A is an essential molecule.

Lipid A is the endotoxically active component of the lipopolysaccharides of Escherichia coli and related Gram-negative bacteria (1, 2). It is also the hydrophobic anchor of lipopolysaccharide that constitutes the outer monolayer of the outer membrane. Structural determination (2–5) has shown that the predominant species of E. coli and Salmonella typhimurium lipid A is a β(1–6)-linked glucosamine disaccharide with (R)-3-hydroxymyristoyl substitutions at positions 2, 3, 2', 3', and phosphate at positions 1 and 4'. The biosynthesis of E. coli lipid A (Fig. 1) has been elucidated (1), and several early enzymes in the pathway have been isolated and partially characterized (1, 6–9).

UDP-N-acetylgalactosamine acyltransferase and lipid A disaccharide synthase (Fig. 1), are coded for by the lpxA and lpxB genes, respectively (10). Both genes have been sequenced (11, 12) and appear to be part of an operon that maps in the minute 4 region of the E. coli chromosome. E. coli strains that contain point mutations in lpxA (11) or lpxB (10, 13) have been isolated and shown to have decreased acyltransferase (11) and disaccharide synthase (6) activity, respectively. However, none of the available mutations results in complete inactivation of enzymatic activity or of lipid A biosynthesis (6, 11, 14). We now describe a new mutant defective in lpxA, in which UDP-N-acetylgalactosamine acyltransferase activity is absent in cell extracts. Growth and lipid A biosynthesis are temperature-sensitive. This mutant provides a model with which to study the effect of acyltransferase deficiency on lipid A levels, outer membrane assembly, and cell physiology.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Media**—All bacterial strains were derivatives of E. coli K12 (Table 1). Cells were grown in LB medium (10 g/liter sodium chloride, 5 g/liter yeast extract, 10 g/liter Tryptone) containing ampicillin (50 μg/ml), tetracycline (20 μg/ml), or diaminopimelic acid (100 μg/ml) where appropriate. Plates contained 15 g/liter agar in the same medium. For the lipid A assay cells were grown in G-56 medium (1/7) which contained 45 mM MES, pH 7.4, 0.3 mM KH₂PO₄, 10 mM potassium chloride, 10 mM magnesium chloride, and 15 mM ammonium sulfate, supplemented with 5 μg/liter thiamine, 0.2% glucose, and 0.4% casamino acids (vitamin-free) (Difco). Phosphate-buffered saline used in neutral Bligh and Dyer (18) lipid extractions contained 8 g/liter sodium chloride. 0.2 g/liter potassium chloride, 1.15 g/liter Na₂HPO₄, 0.2 g/liter KH₂PO₄, pH 7.4.

Ten bacterial mutants with temperature-sensitive lesions near minute 4 were generated by localized mutagenesis (19). P1vir lysates of nitrosoguanidine-treated JW535 (dap⁺) cells were prepared by the method of Silhavy et al. (19). The recipient strain was MF6 (dapD4), and ~20,000 transductants were selected directly on LB plates (which contain negligible amounts of diaminopimelic acid) at 30 °C. Temperature-sensitive transductants were identified by replica plating onto LB agar at 42 °C. Further mapping of the temperature.

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1 The abbreviations used are: LPS, lipopolysaccharide; MES, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; KDO, 3-deoxy-D-manno-octulosonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
FIG. 1. Pathway for biosynthesis of lipid A in E. coli. Evidence for this scheme has been presented previously (1). ACP, acyl carrier protein; U, uridine.

TABLE I

| Strain          | Relevant genotype                      | Source                |
|-----------------|----------------------------------------|-----------------------|
| JW353           | thr-1 leuB6 zae::Tn10 thyA6 met-89     | CGSC                  |
|                 | thi-1 deoC1 lacY1 rpsL67 tonA21        |                      |
|                 | supE44                                  |                       |
| MF6             | dapD4          thr-1 leuB6 hisG4 rpsL67  | Ref. 20               |
|                 | ets-50        thi-1 lacY1 (gal-attl) ara- |                      |
|                 | 14  xyl-5      mtl-1 tsx-78             |                       |
| SM101tet        | dapD*         ipxA2 tetracycline-resistant| This work            |
|                 | transductant of MF6 (NTG-treated JW353 donor) |                     |
| SM101           | dapD*         ipxA2 tetracycline-sensitive| This work            |
|                 | transductant of MF6 (SM101tet donor)   |                       |
| SM105           | dapD*         ipxA* tetracycline-sensitive| This work            |
|                 | transductant of MF6 (SM101tet donor)   |                       |
| RX800           | dapD*         ipxA1 transductant of MF6  | Ref. 11               |
| RX805           | dapD*         ipxA* transductant of MF6  | Ref. 11               |
| W3110           | F- E. coli K12 wild-type, λ             | CGSC                 |
|                 | transductant of MF6 (SM101tet donor)   |                       |

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Sensitive mutations were carried out by transforming the mutants with various plasmids (19) bearing ipxA, ipxB, or other genes near the minute 4 region (10, 11), followed by selection on LB plates at 30 °C and at 42 °C in the presence of the appropriate antibiotics.

Materials—N-Methyl-N'-nitro-N-nitrosoguanidine, deuterated methanol, deuterated chloroform, and MES were purchased from Sigma. UDP-N-acetylb-D-6-[3H]glucosamine was from Du Pont New England Nuclear. Silica Gel 60 thin layer chromatography plates were from E. Merck, Darmstadt, West Germany. Biosafe II liquid scintillation fluid was purchased from Research Products International Corp., Mt. Prospect, IL. Bicinchoninic acid (BCA) was from Pierce Chemical Co. Ca Sep-Paks were from Waters Chromatography Division, Milford, MA. Bio-Sil A Silica gel was from Bio-Rad. Purified lipid IVa, (21) and 32P-labeled lipid IVa, (22) were prepared as described previously. (R)-3-Hydroxymyristic acid was the gift of Drs. L. Anderson and J. Naleway, University of Wisconsin, Madison.

Preparation of Cell Extracts and Enzyme Assays—Cell-free extracts for UDP-GlcNAc acyltransferase and lipid A acylase synthase assays were prepared from stationary phase cells by growing a 75-ml culture overnight at 30 °C in LB broth and harvesting the cells by centrifugation at 3,000 x g for 15 min at 4 °C. Cells were resuspended in 2 ml of 10 mM potassium phosphate, pH 7, containing 5 mM EDTA, and broken by one passage through a French press at 18,000 p.s.i. The suspension was then centrifuged at 4 °C for 60 min at 210,000 x g, and the soluble fraction stored in aliquots at ~80 °C. Protein concentration was determined by the method of Lowry (23) or the BCA method of Smith et al. (24).

Lipid A disaccharide synthase was assayed using a qualitative thin layer assay (9). (R)-3-Hydroxymyristoyl-acyl carrier protein was prepared from E. coli W3110 cells as described by Anderson and Raetz (7). UDP-GlcNAc acyltransferase was initially assayed using 32P-labeled UDP-GlcNAc (120 μM, 6 x 10^4 cpm/μmol), and separating the product and starting material by thin layer chromatography as described previously (7). UDP-GlcNAc acyltransferase was also assayed by a newly developed procedure. Assay mixtures contained [6-3H]UDP-GlcNAc (200 μM, 2 x 10^4 cpm/μmol), (R)-3-hydroxy-myristoyl-acyl carrier protein (50 μM), octyl-D-glucoside (1.0%), HEPES, pH 8.0 (40 mM), fatty acid free bovine serum albumin (10 mg/ml), and enzyme extract containing 50 μg of protein in a final volume of 20 μl. The assay mixture was incubated at 30 °C, and the reaction stopped after 1 or 2 min by diluting in 1 ml of 10 mM HEPES, pH 8.0, and loading directly onto a Ca Sep-Pak equilibrated in 10 mM HEPES pH 8.0. (The Sep-Pak was pre-washed with 10 ml of methanol, followed by 10 ml of water to remove the methanol, before equilibration in the HEPES buffer.) Unreacted starting material was washed through the Sep-Pak with 10 ml of HEPES buffer, and 1-ml fractions were collected and counted in 10 ml of Biosafe I scintillation fluid. The acylated product was then eluted in methanol, and 1-ml fractions were collected and counted in 10 ml of Biosafe II. Unreacted UDP-GlcNAc washed off the Sep-Pak in the first four aqueous fractions, and the acylated product emerged in methanol fractions 1–3. Sep-Paks were regenerated by washing with 10 ml of

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methanol, 10 ml of water and 10 ml of 10 mM HEPES, pH 8.0. 

**Assay for Lipid A in Living Cells**—For the lipid A assay, bacteria from a 30 °C overnight culture were inoculated into fresh G-56 medium at an optical density at 660 nm of 0.05, grown to an optical density of approximately 0.2 at 30 °C, and then temperature-shifted to 42 °C. Lipid A content of the cells was determined by labeling cultures continuously in the presence of 32P (5 μCi/ml in G-56) at 30 °C overnight, during back dilution at 30 °C and following the shift to 42 °C. Rate of synthesis was estimated by pulse labeling identical cultures (25 μCi of 32P/ml) for 5 min either during exponential growth at 30 °C or after 1 h at 42 °C unless otherwise indicated.

32P-Labeled cells were extracted with a single-phase Bligh and Dyer chloroform/methanol extraction (18) by the addition of 2 ml of methanol and 1 ml of chloroform to an 0.8-ml labeled cell culture. Lipopolysaccharide remained associated with the cell debris and was removed by centrifugation at 9200 X g for 15 min. The phospholipid-containing extract was aspirated and kept for subsequent phospholipid determination by thin layer chromatography. The pellet was resuspended by sonication in 1.8 ml of 0.2 M HCl for 30 s (Laboratory Supplies Company Inc., ultrasonic bath) and then hydrolyzed by boiling for 90 min. The “monophosphoryl” lipid A degradation products (5) released from the LPS in the cell debris were extracted into the chloroform phase of a two-phase Bligh and Dyer extract by the addition of 2 ml of chloroform and 7 ml of methanol. Creole E. coli carrier lipid (10 μl of 100 mg/ml solution in chloroform) was added to ensure quantitative recovery. The lower chloroform phase was washed twice with acidic pre-equilibrated upper phase, and 1.6 ml was removed and dried under a stream of nitrogen. The residue was dissolved in a minimum volume of chloroform/methanol (4:1, v/v) and loaded onto a Silica Gel 60 (25 μl) layer plate.

Labeled glycerophospholipids were recovered from the initial single phase extract of the culture, described above, by conversion to a neutral two phase Bligh and Dyer system (18) upon addition of 1 ml of phosphate-buffered saline, 1 ml of chloroform and 10 μl of 100 mg/ml carrier lipid. The lower chloroform phase was washed twice with pre-equilibrated neutral upper phase. A portion of each chloroform phase (10 μl) was loaded onto a silica thin layer plate for layer chromatography.

Lipid A fractions were analyzed initially by TLC in chloroform/pyridine/88% formic acid/water (40:60:16:5, v/v), and phospholipid-containing fractions by TLC in chloroform/methanol/acetic acid/water (25:15:4:2, v/v). Both fractions were also chromatographed in a third solvent system, consisting of chloroform/methanol/water/20% ammonia (40:55:4:2, v/v). Following chromatography, plates were air-dried, bands were visualized by autoradiography, and the appropriate bands were scraped and counted in Bioware II scintillation fluid. The lipid A content was expressed as the ratio of the sum of the monophosphoryl lipid A degradation products to the total glycerophospholipid. The ratio of total lipid A and total glycerophospholipid to the optical density of the culture at 500 nm was also determined.

**Base Hydrolysis**—Base hydrolysis of radiochemical amounts of the acid-released monophosphoryl lipid A fractions was carried out by adding 30 μl of methanol and 2.5 μl of 4.0 M sodium hydroxide to 100 μl of the monophosphoryl lipid A in chloroform, and incubating for 30 min at room temperature. The reaction was neutralized by the addition of 2 ml of 4.0 M hydrochloric acid. The system was converted to a two-phase Bligh and Dyer mixture (18) (final proportions chloroform/methanol/water, 2:2:1:8, v/v). Fresh fatty acids were removed from the lower chloroform phase by passing it through a 1-ml Bio-Sil A silica column in chloroform/methanol (9:1, v/v). The polar base hydrolysis product was eluted from the column in chloroform/methanol (1:1, v/v). The system was then converted to an acid two-phase Bligh and Dyer mixture (18), and the lower phase was washed once with acidic pre-equilibrated upper phase. The final product from the lower phase was analyzed by TLC.

When larger amounts of lipid A and lipid IVa, hydrolysis products were required, the base hydrolysis reaction was scaled up using the same solvents and proportions as already described. For acid and/or base hydrolysis of lipid IVa (typically 5 mg) the solvent volumes used for radiochemical amounts of solvent-extracted cells (see above) were sufficient. However, for large scale acid hydrolysis of LPS, 25 g of frozen E. coli W3110 cells were resuspended in 100 ml of ultrasonicated water. This suspension was converted to a single two-phase Bligh and Dyer mixture by adding water to a total volume of 500 ml, and then 625 ml of chloroform and 1250 ml of methanol. After stirring for 1 h, the insoluble material was collected by centrifugation at 3000 X g for 25 min. The pellet was dried under a stream of nitrogen for several minutes to remove solvent, resuspended in 450 ml of 0.2 M HCl, and boiled in a water bath for 90 min with constant stirring. The acid hydrolysis product was recovered from the chloroform phase after conversion to an acidic two-phase Bligh-Dyer system (18) by addition of 500 ml of chloroform and 500 ml of methanol. After thorough mixing, the material was centrifuged at 2000 X g for 20 min to separate the phases. The lower phase was washed twice with an equal volume of equilibrated acid upper phase before the lower phase was then dried by rotary evaporation at 28 °C. The residue was recovered from the flask by dissolving in 5 ml of chloroform/methanol (9:1, v/v). Base hydrolyzed was usually carried out on 20% of the acid-hydrolyzed material, which was dissolved in 5 ml of chloroform, 1.5 ml of methanol and 125 μl of 4.0 M NaOH, and hydrolyzed for 30 min at room temperature. The hydrolys mixture was neutralized with 125 μl of 4.0 M HCl and converted to an acid Bligh-Dyer system (18) by the addition of 3.5 ml of methanol and 4.25 ml of 0.2 M HCl. The base-hydrolyzed product was separated from liberated fatty acids by elution over a 3-m Bio-Sil A silica column in chloroform/methanol (9:1, v/v). Base-hydrolyzed 4'-monophosphoryl lipid A was washed off the silica in chloroform/methanol (1:1, v/v). The system was converted to an acidic two-phase Bligh and Dyer mixture (18) and the lower phase washed once with acidic pre-equilibrated upper phase. Samples were dried under N2 and analyzed by 1H NMR spectroscopy or FAB mass spectrometry as described below.

**1H NMR Spectroscopy**—1H NMR spectra were obtained on either a Bruker AM-400 (liquid IVa) or AM-500 (E. coli lipid A) spectrometer operating at 400.13 or 600.13 MHz, respectively. The spectrometers were equipped with an Aspect 3000 computer and digital phase shifters. Lipid samples were analyzed as the pyridinium salts which were generated by adding one drop of redistilled pyridine to the purified, free acid form of the lipid dissolved in 1 ml of chloroform, after which the sample was dried under a stream of nitrogen (25). Samples were analyzed in 0.5 ml of CDC13/CD3OD (2:1, v/v). Sample temperature was maintained at 25 °C.

**FAB Mass Spectrometry** (Positive Ion and Negative Ion)—Samples were analyzed at the Middle Atlantic Mass Spectrometry Facility, Johns Hopkins University, Baltimore, MD. FAB mass spectrometry was performed on a Kratos MS-50 mass spectrometer. Samples were dissolved in chloroform/methanol (2:1) and applied to the probe in either thioglycerol (for the positive mode) or glycerol/thioglycerol (1:1) (for the negative mode). The spectrometer ion source was operated at 8 kV. Mass spectra were obtained by scanning the mass ranges of 28-1500 (positive ions) or 126-1166 (negative ions) at a rate of 30 s/decade at room temperature. Data were collected on a Kratos DS-55 data system.

RESULTS

Isolation of a Mutant Defective in UDP-N-acetylgalacto-



UDP-GlcNAc Acyltransferase Mutant of E. coli
N-acetylglucosamine acyltransferase activity was measured in cell extracts of SM101, RX800, and the isogenic wild-type by the method of Anderson and Raetz (7). Unreacted \[^{14}C\]UDP-GlcNAc was separated from the product by thin layer chromatography on Silica Gel.

Cell growth was followed by measuring the absorbance of SM101 in LB and plating viable cells, expressed as colony-forming units (CFU)/ml, were determined by diluting cultures of SM101 in G-56 medium and shifted from 30 to 42 °C at time 0. Cell growth was followed by measuring the absorbance of SM101 (IpxA+ and IpxA') at 2 and 10 min. Autoradiography was for 12 h at room temperature.

With the new assay, no activity was detected in SM101.

To determine whether the IpxA2 mutation caused cell death under nonpermissive conditions, or merely arrested cell growth, cells of SM101 growing exponentially at 30 °C on LB medium were shifted to 42 °C. The optical density of the culture measured at 550 nm plateaued 2 h after the shift, and then slowly declined (Fig. 3). However, cell viability decreased several orders of magnitude starting 1 h after the temperature shift (Fig. 3) as judged by plating efficiency on LB agar at 30 °C. Loss of viability was also observed when SM101 was shifted to 42 °C on minimal G-56 medium (data not shown).

The IpxA2 mutation did not cause any gross abnormalities in the morphology of the outer membrane. After 1 h at 42 °C, cells of mutant SM101 were indistinguishable from wild-type, as determined by phase contrast and scanning electron microscopy (data not shown).

Correction of the Enzymatic Defect and Temperature Sensitivity of SM101 by Various Hybrid Plasmids—Hybrid plasmids encoding fragments of the minute four region of the E. coli chromosome were tested for their ability to correct the temperature sensitivity and the enzymatic defect of SM101. The previously described plasmids pDC2, pCR9, pDC29, pDC24, and pLPXA (10, 11) were used to transform SM101. Except for pDC24, all of the plasmids relieved the temperature-sensitive phenotype of SM101. The four plasmids that restored the growth of SM101 at 42 °C all harbor the IpxA gene. pDC24 contains the gene lpxB, which codes for the disaccharide synthase, but only contains a small portion of the adjacent lpxA gene. pLPXA bears the lpxA gene as its only insert (11), and this plasmid restores the wild-type level of UDP-N-acetylglucosamine acyltransferase to SM101 (data not shown). Taken together, these results demonstrate that the lesion in SM101 that confers temperature sensitivity is in the same gene (lpxA) encoding the acyltransferase, and that the lpxA gene is essential for growth.

Extraction and Quantitation of Lipid A—The temperature sensitivity of strains harboring mutations in the gene coding for UDP-GlcNAc acyltransferase raised several important questions. Is UDP-GlcNAc acyltransferase the sole committed enzyme responsible for the generation of lipid A in E. coli? What is the rate of lipid A synthesis and the content of lipid A in mutant and isogenic wild-type cells at 30 and 42 °C? Can cells lacking lipid A grow under some conditions?

To answer these questions it was necessary to devise a simple, new assay for determining the content of lipid A in small samples of radiolabeled cultures. The existing methods for extracting LPS with phenol-containing solvents are not necessarily quantitative (15, 16). Because of the structure and microheterogeneity of intact LPS (2) there is no unique radiochemical precursor with which to label and quantitate the number of lipid A molecules in cells.

We exploited the acid lability of LPS (1, 2) in order to quantify the amount of lipid A present. As shown by Takayama and coworkers (5), mild acid hydrolysis of isolated LPS cleaves the KDO-lipid A linkage and removes the anomeric phosphate. Partial loss of esterified fatty acids may also occur, accounting for the heterogeneity (5) of the observed products (Fig. 4). All lipid A degradation products prepared by acid hydrolysis of E. coli lipid A. Diagram shows predicted products of acid hydrolysis, followed by base hydrolysis, of the E. coli lipid A moiety of LPS. R, R-3-hydroxymyristate; R', lauroxyloxymyristate; R", myristooyxymyristate.
hydrolysis, however, retain the 4'-monophosphate function, and are therefore referred to as "4'-monophosphoryl lipid A species" (3-5). These substances can be separated from glycerophospholipids, and from each other, by thin layer chromatography. Unlike intact LPS, they can be extracted quantitatively with chloroform/methanol/water mixtures using standard Bligh-Dyer proportions (18). The phosphorus content of the combined 4'-monophosphoryl lipid A units is an accurate measure to the molar amount of lipid A present in a LPS sample.

In a typical experiment, exponentially growing E. coli were either labeled uniformly or pulsed with 32P, as described under "Experimental Procedures." A sample of the culture (0.8 ml) was quenched with 2 ml of methanol and 1 ml of chloroform, yielding a single phase Bligh-Dyer mixture. The glycerophospholipids were extracted into the solvents, but the LPS remained associated with the cell debris and was collected by centrifugation. Next, the cell debris was resuspended in aqueous 0.2 M HCl and boiled for 90 min. The 4'-monophosphoryl lipid A was then recovered by a second Bligh-Dyer extraction. Time-course studies (not shown) revealed that most of the 4'-monophosphoryl lipid A was released from the cell debris in 30 min and that the recovery did not decline with prolonged hydrolysis. A portion of both the glycerophospholipids and the 4'-monophosphoryl lipid A species were then analyzed by thin layer chromatography and autoradiography, as shown in Fig. 5 for cells that had been uniformly labeled. The glycerophospholipid extract (lane 2) did not contain significant amounts of the 4'-monophosphoryl lipid A species, and the latter were free of glycerophospholipids (lane 1). The ratio of lipid A to glycerophospholipid (determined by quantitation of the 32P in each fraction) in uniformly labeled, wild-type cells was approximately 0.12, consistent with one monolayer of lipid A in each cell. The same ratio was observed when wild-type cells were pulse-labeled with 32P, for only 5 min.

The above assay is applicable to all smooth strains and to rough strains of the Rb and Ra chemotypes (data not shown). Because deep rough LPS is soluble in chloroform/methanol mixtures it is removed from the cells together with glycerophospholipids. Consequently the above assay cannot be used with strains of the Re, Rd, and Rc chemotypes (2).

Characterization of the 32P-Labeled Lipid A Fragments Released from Cells by Acid Hydrolysis—The lipids released from solvent-extracted E. coli by acid hydrolysis appeared similar to the monophosphoryl lipid A degradation products obtained from purified S. typhimurium LPS (5) as judged by their mobility on thin layer silica plates. We also characterized their relationship to the standard compound, lipid IVα (Fig. 1), a tetraacyl disaccharide-1,4'-bisphosphate precursor of mature lipid A that can be purified from KDO-deficient mutants of S. typhimurium (21). When 4'-32P-lipid IVα (22) was acid hydrolyzed and compared to the lipids released by acid hydrolysis of solvent-extracted E. coli, the 4'-32P-lipid IVα-derived material migrated more slowly than the major cell-derived bands (Fig. 6, lanes 2 and 4). This observation is consistent with the proposed structures (Figs. 1 and 4), since acid hydrolysis should remove mainly the anomeric phosphate of lipid IVα, leaving a tetraacyl-disaccharide 4'-monophosphate. The corresponding derivative of mature lipid A would contain some normal fatty acids in acyl-oxyacyl linkage, in addition to the (R)-3-hydroxy-myristoyl residues attached to the disaccharide backbone (Fig. 4). When the acid-hydrolyzed compounds were hydrolyzed further with mild alkali, however, both the lipid IVα-derived, and the cell-derived substances collapsed to identical, more slowly migrating species (Fig. 6, lanes 3 and 5). This observation is expected, since removal of all the O-linked fatty acid chains by mild base leaves only two N-linked hydroxy fatty acids and the 4'-phosphate on the

![Fig. 5. Comparison of glycerophospholipids and 4'-monophosphoryl lipid A degradation products by TLC.](http://www.jbc.org/)

![Fig. 6. TLC analysis of compounds obtained by sequential acid and base hydrolysis of E. coli lipid A and lipid IVα.](http://www.jbc.org/)
UDP-GlcNAc Acanthobacteriaceae Mutant of E. coli

disaccharide backbone in both samples (Fig. 4).

The chemical basis for the apparent doublet after sequential acid and base hydrolysis observed in both samples (Fig. 6) is uncertain.

**FAB Mass Spectrometry and 1H NMR Spectroscopy of the Products of Sequential Acid-Base Hydrolysis**—The products of sequential acid-base hydrolysis of solvent-extracted E. coli, and pure lipid IVα were compared by fast atom bombardment mass spectrometry in the positive and negative modes. The results indicate that the material derived from E. coli (Fig. 6, lane 3) is very similar to the comparable hydrolysis product of lipid IVα (Fig. 6, lane 5). Only one major species appeared in both samples in the negative mode at m/z 571 (not shown), and this is attributed to [M – H]−, consistent with the structure shown at the bottom of Fig. 4. In the positive mode, the major species of the highest molecular weight were common to both preparations (Fig. 7). The peak at m/z 895.5 in the positive mode can be accounted for by [M + Na]+. The other major peak in the positive mode at m/z 468.3 is probably the oxonium ion derived from the nonreducing end, which arises by cleavage of the glycosidic linkage. The peak at m/z 550.6 has not been identified conclusively, but it is present in both products. The smaller fragments (below m/z 468) present in the E. coli-derived sample have not been identified but are presumed to derive from minor impurities that copurified with the monophosphoryl lipid A species from the whole cell suspensions.

The results of the FAB mass spectrometry confirm the conclusions of the radio-chromatographic analysis (Fig. 6), that sequential acid-base hydrolysis of solvent-extracted E. coli and of pure lipid IVα generate the same predominant 4'-monophosphoryl lipid A species (Fig. 7). The identity of these materials was also confirmed by 1H NMR spectroscopy (Fig. 8). Both preparations were converted to their pyridinium salts and dissolved in 0.6 ml of CDCl3/CD3OD (2:1, v/v). The major features of their spectra (Fig. 8) are very similar, consistent with the scheme shown in Fig. 4.

In a separate experiment, the lipids released by acid hydrolysis of solvent-extracted E. coli (Fig. 6, lane 2) were analyzed directly by FAB mass spectrometry, without purification or additional base hydrolysis. In the negative mode (data not shown), prominent mass ions were observed at m/z 1717 and m/z 1507. These correspond to a hexacylated 4'-monophosphoryl lipid A bearing laurate and myristate as the normal fatty acyl moieties, and to a pentaacylated 4'-monophosphoryl lipid A bearing only laurate as its sole normal (non-hydroxy) fatty acyl chain. The simplicity of the mass spectrum of this crude monophosphoryl lipid A preparation further validates our method for the determination of the lipid A content of E. coli.

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**Fig. 7. Partial fast atom bombardment mass spectra of the substances generated by sequential acid and base hydrolysis of solvent-extracted E. coli or lipid IVα.** FAB spectra of acid- and base-hydrolyzed lipid IVα (A) and E. coli derived substances (B) are shown in the positive mode.
UDP-GlcNAc Acyltransferase Mutant of E. coli

A

B

FIG. 8. 'H NMR analysis of the substances generated by sequential acid and base hydrolysis of solvent-extracted E. coli or lipid IVα. Samples were prepared as the pyridinium salts, and spectra were recorded in 0.6 ml of CDCl3/CD3OD (2:1, v/v) at 400.13 MHz (lipid IVα, panel A) or 500.13 MHz (E. coli lipid A, panel B). The chemical shifts are expressed as parts per million relative to an internal standard of tetramethylsilane.

Lipid A Content of Acyltransferase-deficient Mutants—The above data indicates that the lipids released by acid hydrolysis of solvent-extracted E. coli are a mixture of 4'-monophosphoryl lipid A fragments. Depending on the time of labeling with 32P, the 4'-monophosphoryl lipid A species prepared in this manner can provide a measure of the total lipid A content or the rate of lipid A synthesis. The amount of 32P in the monophosphoryl lipid A fraction can be normalized to the A_{230} or to the 32P in the total glycerophospholipid fraction. Cells were usually labeled in G-56 medium, which has a lower phosphate content than LB, allowing greater incorporation of 32P into all lipids.

As shown in Fig. 9, the banding pattern of the [32P]monophosphoryl lipid A species, recovered after pulse-labeling the cells, is the same in both the wild-type and mutant, indicating that the lipid A produced by the mutant is not structurally different from that of the wild-type. The same results are obtained with continuous labeling (data not shown). The rate of synthesis of all the labeled species derived from SM101 is reduced to a similar extent after 1 h at 42 °C (Fig. 9), consistent with a lesion in the first step of the biosynthetic pathway.

The lipid A content of the temperature-sensitive, acyltransferase-deficient mutant (SM101) and the isogenic wild-type (SM105) were compared at 30 °C and after 1 h at 42 °C. The ratio of lipid A to phospholipid in wild-type strains (such as SM105) is consistently around 0.12 at both temperatures. In the mutant SM101 the lipid A to phospholipid ratio was 0.08 at 30 °C (the permissive temperature), and it dropped to 0.05 after 1 h at 42 °C (the nonpermissive temperature).

Fig. 10 shows the results of 5 min pulse labeling of SM105 and SM101 with 32P, at 30 °C (time 0) and as a function of time following a shift to 42 °C. Lipid A and phospholipid syntheses rise in the wild-type at 42 °C when normalized to the A_{230} until the cells reach stationary phase (Fig. 10, A and B). The ratio of lipid A to phospholipid in pulse-labeled SM105 is around 0.14 at all times (Fig. 10C), approximately the same as the ratio seen with continuous labeling (0.12). In mutant SM101, lipid A synthesis fails to rise in the first 5 min after shifting from 30 to 42 °C, and then it gradually drops (Fig. 10A). Phospholipid synthesis, however, does rise in SM101 after the shift, as in the wild-type (Fig. 10B), even though growth in SM101 is inhibited and viability begins to decline after 60 min (Fig. 3). Fig. 10C shows that the ratio of lipid A to phospholipid synthesis in SM101 falls rapidly in the first 20 min after temperature shift, while the ratio for the wild-type strain is unaffected. From these results we conclude that there is a 10-fold inhibition of the rate of lipid A synthesis in the mutant at 42 °C relative to the rate of glycerophospholipid synthesis.

Effect of Restoration of Acyltransferase Activity on Lipid A Biosynthesis—The presence of a plasmid encoding the ipxA gene in SM101 (SM101/pLPXA) restored temperature resistance and UDP-N-acetylglucosamine acyltransferase activity as noted above. SM101/pLPXA was also assayed for the rate of lipid A synthesis, as in Fig. 10 (data not shown). In SM101/
Fig. 10. Rate of synthesis of lipid A, and glycerophospholipids after a shift to 42°C. SM105 (lpxA+) and SM101 (lpxA2) were grown in G-56 medium to A600 of approximately 0.2, then shifted from 30 to 42°C at time 0. Samples were removed at the times indicated and pulse-labeled for 5 min with 32P. A, lipid A (counts/min/0.8-ml culture) to A600 ratio; B, glycerophospholipid (counts/min/0.8-ml culture) to A600 ratio; C, lipid A to glycerophospholipid ratio.

pLPXA the lipid A to phospholipid ratio was 0.12 after 1 h at 42°C, comparable to that of wild-type strains, and 10-fold higher than SM101.

Isolation of Temperature-resistant Revertants of SM101—The reversion rate of SM101, as judged by growth of colonies at 42°C on LB agar, was approximately 3 × 10⁻⁷. Ten spontaneous temperature-resistant revertants of SM101 were isolated and assayed for lipid A synthesis in vivo and UDP-N-acetylglucosamine acyltransferase activity in vitro. Two revertants regained measurable acyltransferase activity (0.15 and 0.24 nmol min⁻¹ mg⁻¹), but no revertants regained wild-type levels (1.8 nmol min⁻¹ mg⁻¹) (Table II). The lipid A/phospholipid ratios of these revertants were 0.10 and 0.12, respectively, indicating that lipid A biosynthesis was also restored. One revertant regained lipid A (lipid A/phospholipid ratio 0.10) even though restoration of the in vitro acyltransferase activity could not be detected. When Plvir lysates of each of the three revertants were transduced into MF6 (dapD), all of the resulting dapD+ colonies were temperature-resistant. In contrast, when a lysate of SM101 was used to transduce MF6, half of the dapD+ colonies were temperature-sensitive. This suggests that the revertant that appears to lack measurable UDP-GlcNAc acyltransferase may, in fact, have a subtle bypass mutation within the lpxA gene. A larger number of revertants will have to be analyzed in more detail in order to determine whether or not extragenic bypass mutations of lpxA2 can be isolated.

DISCUSSION

In previous studies (7), we have described the existence of a novel enzyme in E. coli extracts that catalyzes the transfer of an (R)-3-hydroxyacyl moiety from (R)-3-hydroxyacyl-acyl carrier protein to UDP-GlcNAc (Fig. 1). The extreme specificity of the enzyme for acyl chain length and for an (R)-3-hydroxy function (7) is in accord with the fatty acid composition of E. coli lipid A (1, 2). The inability of the enzyme to utilize (R)-3-hydroxyacyl-coenzyme A (7) may explain why exogenous (R)-3-hydroxybutyrate is not incorporated into lipid A by living cells (20). Although the biochemical features of UDP-GlcNAc acyltransferase strongly suggest that it plays a key role in lipid A biosynthesis in vivo, analogous to that of glycerol-3-phosphate acyltransferase in glycerophospholipid metabolism (27), conditional mutants in UDP-GlcNAc acyltransferase have not been reported.

In the course of mapping a mutant defective in the lipid A disaccharide synthase (Fig. 1), encoded by the lpxB gene near minute 4 on the chromosome (10), we have previously demonstrated that the structural gene for UDP-GlcNAc acyltransferase (designated lpxA) maps just upstream of lpxB (10, 11). Both lpxA genes are part of a complex operon that includes at least three additional open reading frames of unknown function, and dnaE, the catalytic subunit of DNA polymerase III (12, 28). The entire operon has been sequenced, but its biological significance remains uncertain (12, 28). Both lpxA gene products have been overexpressed (10) and purified to homogeneity (9).² NH₂-terminal protein sequencing has confirmed that lpxA and lpxB are structural genes (9-12).

Since efficient methods for supplementing E. coli with exogenous phospholipids or lipid A precursors are not available (27), we have generated conditional mutants in UDP-GlcNAc acyltransferase using localized chemical mutagenesis (19). Of ~20 temperature-sensitive mutations mapping near minute 4, as demonstrated by cotransduction with the dapD locus, two are defective in lpxA. Strain RX800 (lpxA1) (11) possesses considerable residual activity and has an unacceptable high reversion rate (~5 × 10⁻⁶). RX800 has not been characterized further. Mutant SM101 (lpxA2) contains no measurable residual activity at any temperature, has a lower spontaneous reversion frequency (~3 × 10⁻⁷), and is strikingly defective in lipid A biosynthesis in vivo at 42°C. All of the biochemical and phenotypic alterations of SM101 are corrected by plasmids bearing lpxA (11) alone. Since the rate of lipid A biosynthesis in SM101 at 42°C is inhibited ~10-fold relative to wild-type (Fig. 10), we conclude that over 90% of the lipid A of wild-type E. coli is generated via the UDP-GlcNAc acyltransferase pathway. To demonstrate that all lipid A molecules arise by the action of UDP-GlcNAc

²K. Radika, unpublished data.
acyltransferase, it will be necessary to generate "tighter" alleles of lpxA, preferably ones that are under the control of a regulated promoter (29).

The temperature sensitivity of SM101 strongly suggests that lipid A is an essential molecule for the growth and replication of E. coli, most likely, because it is required for outer membrane assembly (1, 2). Outer membrane proteins appear to have a high affinity for the lipid A domain of LPS (30). It will be of considerable interest to study the synthesis and export of outer membrane proteins when lipid A synthesis is inhibited. The rapid loss of viability of SM101 at 42 °C (Fig. 3) may be significant in this regard, since the accumulation of functional porin trimers within the inner membrane might be lethal. Inhibition of protein synthesis has no immediate effect on the synthesis and export of LPS (26).

In the coming years, it will be important to examine a large collection of temperature-resistant revertants of SM101. The few spontaneous revertants that we have studied so far (Table II) appear to regain lipid A, most likely, because they have regained sufficient UDP-GlcNAc acyltransferase function to support a normal rate of lipid A synthesis in vivo. It is conceivable, however, that cells could grow without lipid A, or possibly even without an outer membrane, provided that appropriate bypass mutation(s) are present. It may also be possible to bypass the requirement for UDP-GlcNAc acyltransferase by inducing an alternative pathway for lipid A synthesis, by activating a gene that codes for an isoenzyme, or by altering the regulation of the lpxA/dnaE operon. The simple radiochemical assay for lipid A synthesis and content that we have devised will facilitate the analysis of additional mutants and bypass revertants.

The observation that mutants defective in UDP-GlcNAc acyltransferase lose viability under nonpermissive conditions (Fig. 3) suggests that inhibitors of the early steps of the lipid A pathway (Fig. 1) might have utility as antibiotics. Inhibitors of CMP-KDO biosynthesis have been explored in this context (31, 32), but so far the available compounds lack potency and are not bacteriocidal. Nevertheless, the gram-negative species of the CMP-KDO synthesis inhibitors (31, 32) validates the generality of LPS biosynthesis as a pharmacological target.

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