A Triple Mutant of *Escherichia coli* Lacking Secondary Acyl Chains on Lipid A*

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All possible combinations of insertion mutations in the three genes encoding the acyl carrier protein-dependent late acyltransferases of lipid A biosynthesis, designated *lpxL*(*htrB*), *lpxM*(*msbB*), and *lpxP*, were generated in *Escherichia coli* K12 W3110. Mutants defective in *lpxM* synthesize penta-acylated lipid A molecules and grow normally. Strains lacking *lpxP* fail to incorporate palmitoleate into their lipid A at 12 °C but make normal amounts of hexa-acylated lipid A and are viable. Although *lpxL* mutants and *lpxL* *lpxM* double mutants grow slowly on minimal medium at all temperatures, they do not grow on nutrient broth above 32 °C. Such mutants retain the ability to synthesize some penta- and hexa-acylated lipid A molecules because of limited induction of *lpxP* at 30 °C but not above 32 °C. MKV15, an *E. coli* *lpxL* *lpxM* *lpxP* triple mutant, likewise grows slowly on minimal medium at all temperatures but not on nutrient broth at any temperature. MKV15 synthesizes a lipid A molecule containing only the four primary (R)-3-hydroxymyristoyl chains. The outer membrane localization and content of lipid A are nearly normal in MKV15, as is the glycerophospholipid and membrane protein composition. However, the rate at which the tetra-acylated lipid A of MKV15 is exported to the outer membrane is reduced compared with wild type. The integrity of the outer membrane of MKV15 is compromised, as judged by antibiotic hypersensitivity, and MKV15 undergoes lysis following centrifugation. MKV15 may prove useful as a host strain for expressing late acyltransferase genes from other Gram-negative bacteria, facilitating the re-engineering of lipid A structure in living cells and the design of novel vaccines.

The lipid A component of the lipopolysaccharide in Gram-negative bacteria usually contains four “primary” (R)-3-hydroxyacyl chains linked directly to the 2-, 3-, 2’-, and 3’-positions of the glucosamine disaccharide backbone (see Fig. 1 of accompanying article (8) and Refs. 1–3). Some of the (R)-3-hydroxy groups are further modified with “secondary” acyl chains, forming acyloxyacyl moieties (1–3). In wild type *Escherichia coli* and *Salmonella typhimurium*, secondary lauroyl and myristoyl groups are attached to the (R)-3-hydroxymyristoyl chains located at the 2’- and 3’-positions, respectively, of the distal glucosamine (see Fig. 1 of accompanying article (8) and Refs. 1–3).

The “late acyltransferases” of *E. coli* that are responsible for the incorporation of laurate and myristate (4–6) are designated LpxL and LpxM (see Fig. 1 of the accompanying article (8)). They are located in the inner membrane, and their active sites must be oriented toward the cytoplasm, because they require acyl-acyl carrier proteins (ACPs)1 as donor substrates (4–6). An additional ACP-dependent late acyltransferase, LpxP, is induced upon cold shock (12 °C) (see Fig. 1 of accompanying article (8) and Ref. 7). LpxP incorporates the unsaturated fatty acid palmitoleate in the place of laurate (7). An insertion mutation in *E. coli* *lpxP* abolishes the incorporation of palmitoleate into lipid A at 12 °C but does not inhibit growth (8); however, it increases the susceptibility of the cells to some antibiotics at low growth temperatures (8).

When *Salmonella* or *E. coli* cells are grown under conditions that activate the PhoP/PhoQ two-component regulatory system (9–11) or in the presence of ammonium metavanadate (12), 25–50% of their lipid A is further acylated with a secondary palmitic acid chain at position 2 on the proximal glucosamine (see Fig. 1 of accompanying article (8)). Palmitate is transferred to hexa-acylated lipid A in the outer membrane by the unusual acyltransferase PagP (11). The latter uses glycerophospholipids as its acyl donor (11, 13). PagP shares no sequence similarity with the acyl-ACP-dependent late acyltransferases (5–7, 11).

The *lpxl* gene was discovered by Georgopoulos and co-workers (14, 15) as being required for rapid growth above 32 °C on nutrient broth and was initially designated *htrB* for “high temperature requirement gene type B.” Clementz et al. (5, 16) subsequently elucidated the function of HtrB as the lauroyltransferase (*LpxL*) of lipid A biosynthesis. Further analysis of *lpxl*(*htrB*) mutants revealed that tetra-acylated lipid A species accumulate in their inner membranes when cells are shifted to 42 °C (17), suggesting that tetra-acylated species are exported inefficiently. As shown in the present study, the ability of *lpxl* mutants to grow at 30 °C on nutrient broth is explained by the limited induction of *lpxP* at 30 °C (but not at 42 °C) when *lpxl* is deleted, permitting the synthesis of some penta- and hexa-acylated lipid A species at 30 °C in *lpxl* mutant strains.

Two additional genes, *msbA* and *msbB*, were identified by Karow and Georgopoulos (18, 19) based on their ability to suppress the temperature-sensitive growth of *lpxl* mutants when provided in multiple copies. The *msbA* gene encodes an ABC transporter (19–21) and is necessary for the export of all

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1 The abbreviations used are: ACPs, acyl carrier proteins; Kdo, 3-deoxy-d-manno-octulosonic acid; MALDI/TOF, matrix-assisted laser desorption/ionization/time-of-flight; LPS, lipopolysaccharide.
late acyltransferases of strains with insertion mutations in the three ACP-dependent late acyltransferases of E. coli (see Fig. 1 of accompanying article (8) and Ref. 6). Overexpression of LpxM is presumed to compensate for the absence of LpxP by permitting the transfer of myristate to the precursor Kdo4-lipid IVα in the absence of laurate (see Fig. 1 of accompanying article (8)), which is normally incorporated prior to myristate (6). E. coli mutants lacking LpxM but containing lpxL are not temperature-sensitive for growth, because they make a lipid A species that is fully penta-acylated, which is rapidly exported (17, 23). However, LpxM mutants are characterized by greatly attenuated cytokine induction and reduced virulence in some animal models (23–25).

We now report the construction of all possible combinations of strains with insertion mutations in the three ACP-dependent late acyltransferases of E. coli. Care was taken to avoid second site suppressors by performing strain constructions on minimal medium (14). As anticipated from the biochemistry (see Fig. 1 of accompanying article (8)), these mutants produce lipid A species with varying acylation states. A triple mutant (MKV15) lacking all three ACP-dependent late acyltransferases produces a lipid A species containing only the four primary (R)-3-hydroxyxymyristate residues but is viable on minimal medium at all temperatures. PagP is not induced as a compensatory mechanism under any of the conditions that we have examined.

**EXPERIMENTAL PROCEDURES**

**Materials—**32P ([~900 Ci/mmol] and phosphatidylcholine [t-[1-14]C] (dipalmitoylphosphatidylcholine, ~100 mCi/mmol) were purchased from PerkinElmer Life Sciences. The bincholonic acid protein determination kit was from Pierce. Sodium phosphate dibasic, ammonium sulfate, succrose, 88% formic acid, and glass-backed Silica Gel-60 thin layer chromatography plates (0.25 mm) were from Merck. Pyridine, ammonium acetate, and other bulk chemicals were from Mallinckrodt Chemical Works. Granulated agar, yeast extract, tryptone, and Bacto25° agar used for minimal medium plates were from Difco. A P1vir bacteriophage stock was kindly provided by Dr. R. Webster of Duke University. All other materials were purchased from Sigma unless otherwise mentioned.

**Bacterial Strains and Growth Conditions—**Table I shows the E. coli K12 strains and plasmids used in this study. Cells were cultured at 30°C in a supplemented phosphate-buffered minimal medium ("Kozak" medium), consisting of 67 mM Na2HPO4, 46 mM KH2PO4, 18 mM NaCl, 7.5 mM MgCl2, 54 mM glucose, 1.5 mM K2HPO4, 4.5 mM FeSO4, and 0.075 mM thiamine (26). In some experiments cells were grown on modified G56 minimal medium, which contains 45 mM HEPES, pH 7.4, 0.3 mM KH2PO4, 10 mM KCl, 10 mM (NH4)2SO4, 0.2% glucose, 1.7 mM MgSO4, 0.117 mM CaCl2, 0.015 mM FeSO4, and 0.075 mM thiamine (27). Cells were sometimes grown on LB broth, which contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter (28). Antibiotics were added as necessary at 30 μg/ml for kanamycin, 25 μg/ml for chloramphenicol, or 25 μg/ml for tetracycline.

**P1vir Bacteriophage Transduction of the lpxP::kan Insertion Mutation into Strains Containing lpxL and/or lpxM Mutations—**A P1vir bacteriophage lysate of donor strain MKV11 was made using the protocol of Miller (28). Overnight cultures of the recipient strains were resuspended in MC buffer (0.1 mM MgSO4 and 5 mM CaCl2) and infected with dilutions of the lysate at 30°C for 30 min. The transduction mixture was plated onto LB broth agar containing 30 μg/ml kanamycin and 5 mM sodium citrate or, in the case of recipient strains harboring the lpxL mutation, onto minimal A plates (28) containing 30 μg/ml kanamycin to prevent the accumulation of second site suppressor mutations. Colonies appearing after 1–2 days of growth at 30°C were then purified and tested for all relevant antibiotic resistance patterns, growth phenotypes, and temperature sensitivity.

**Growth Rate of Late Acyltransferase Mutants in Minimal Medium or LB Broth—**Stationary phase cultures of all seven late acyltransferase mutants and the wild type strain, W3110, were grown in Kozak medium (26) with antibiotics at 30°C. These were used to inoculate eight individual 6-ml cultures of Kozak medium without antibiotics to an optical density (A600) of 0.01. The A600 of the cultures was monitored into late log phase using a Milton-Royal Spectronic 21D spectrophotometer. The cultures were diluted as needed to give readings of less than 0.6 within the linear range of the spectrophotometer. To determine growth rate in LB broth, 6-ml cultures of cells were grown in Kozak medium to A600 = 0.1, collected at 2000 × g for 1 min in a Beckman JS4.3 rotor, and resuspended in 6 ml of Luria broth. The A600 was again monitored until the wild type cells entered stationary phase.

**32P Labeling and Isolation of Lipid A—**Five-ml cultures of the various late acyltransferase mutants and wild type E. coli were grown at 30°C in modified G56 medium (27) to A600 = 0.4. Next, 32P (5 μCi/ml of cultures added; and the cells were grown for an additional 120 min to A600 = 1.0. A small scale lipid A isolation was performed directly on 1.6 ml of each cell culture as described previously (12), but without first harvesting the cells. Briefly, the glycolipidophospholipids were extracted with a single-phase Bligh-Dyer mixture (29), generated by adding 4 ml of methanol and 2 ml of CHCl3, to a 1.6-ml culture. After centrifugation and removal of the supernatant, the cell residue was hydrolyzed in 1.8 ml of 12.5 mM sodium acetate buffer, pH 4.5, and 1% SDS at 100°C to cleave the Kdo-lipid A linkage (12, 30). The various lipid A 1,4-bisphosphate and lipid A 1-pyrophosphate species were then recovered by conversion of the suspension to a two-phase Bligh-Dyer system (12, 31). The lower phase (containing the total cellular lipid A and small amounts of decomposition products) was dried under nitrogen. The dried samples were dissolved in 100 μl of chloroform/methanol (4:1, v/v), and 10 μl of each was spotted onto a 20 × 20-cm silica gel TLC plate, which was developed with the solvent chloroform, pyridine, 88% formic acid, water (50:50:16.5, v/v). The plate was exposed to a PhosphorImager screen, and the radioactivity was quantified with a Molecular Dynamics PhosphorImager, equipped with ImageQuant version 1.2 software for the Macintosh.

**Large Scale Isolation of Lipid A from Cells—**Typically, 100-ml cultures were grown using Kozak medium at 30°C to A600 = 1.0. Cells were collected by centrifugation at 5000 × g and resuspended in 40 ml of phosphate-buffered saline (32). Glycolipidophospholipids were extracted with a single phase Bligh-Dyer mixture, as described above, and the lipid A was recovered from the cell residue by a second Bligh-Dyer extraction following hydrolysis at 100°C in sodium acetate buffer, pH 4.5, and 1% SDS (12). The lipid A was then purified by chromatography on DEAE-cellulose columns as described previously (12, 31).

**Mass Spectroscopy of Lipid A Samples—**Spectra were acquired in the negative-ion linear mode using a Kratos analytical (Manchester, UK) matrix-assisted laser desorption ionization/time of flight (MALDI/TOF) mass spectrometer at nitrogen laser, 2376 eV nitrogen ionization voltage, and time-delayed extraction. Each spectrum was averaged over 50 scans. The lipid A samples were prepared for MALDI/TOF analysis by depositing 0.3 μl of the sample dissolved in chloroform/methanol (4:1, v/v), followed by 0.3 μl of the matrix, which was a mixture of saturated 6-aza-2-thiothiophene in 50% acetonitrile and 10% trisaccharidic ammonium citrate (9:1, v/v). The sample mixtures were allowed to dry under nitrogen prior to mass analysis. Hexa-acylated lipid A 1,4-bisphosphate from wild type E. coli (Sigma) was used as an external standard for calibration.

**Separation of the Inner and Outer Membranes of 32P-Labeled Wild Type and Triple Mutant Cells—**Wild type W3110 and the triple lpxL lpxP lpxM mutant, MKV15, were grown to A600 = 0.7 in 100 ml of modified G56 minimal medium (27). Cultures were split into two equal portions, and the cells were collected at 2000 × g for 15 min in a Beckman JS4.3 rotor at room temperature. One portion was resuspended in 100 ml of modified G56 minimal medium and the other in 100 ml of LB broth. The resuspended cells were incubated with shaking for 10 min at 30°C. Next, 32P (5 μCi/ml) was added to each of the cultures, which were then incubated with shaking for another 10 min at 30°C. The cells were collected by centrifugation at 8000 × g in a Beckman JA14 rotor at 4°C and immediately frozen at −80°C as pellets. Care was taken not to disturb the cell pellet while the medium was being decanted, as some of the mutants were easily lysed at this state. The frozen pellets were then thawed in 10 ml of 10 mM Tris acetate, pH 7.8, and 25% sucrose, treated with 0.1 mg/ml lysozyme and 1.0 mM EDTA (33), and subjected to sonic irradiation in an ice bath at Output Control 8 and Duty Cycle 1.5 on a Branson Sonifier 250 for 15 s. Doubly washed cell membranes were isolated by ultracentrifugation at 150,000 × g in a Beckman Ti-70 rotor and resuspended in 2 ml of 10 mM Tris acetate, pH 7.8, and 25% sucrose. Inner and outer membranes were then separated on a 12-ml gradient of 30–60% (w/w) sucrose in 10 mM Tris acetate, pH 7.8, at 4°C by ultracentrifugation at 155,000 × g using a Beckman SW-41 swinging bucket rotor (17, 22, 33).
Overexpression of lpxP and lpxM with the T7lac System—The lpxP structural gene was cloned and the protein overexpressed as described in the preceding paper (8). The lpxM structural gene (18) was obtained by PCR using W3110 genomic DNA as the template. Primers specific for lpxM containing an NdeI restriction site at the 5′ end (5′-GCCGCCGCATGATGAGAAGCGAAAAAA-3′) and a BamHI site at the 3′ end (5′-GCCGCCGGATCCTTTGATGAGGATA-3′) were used. The PCR product was digested with NdeI and BamHI and ligated into the similarly digested vector, pET21a (Novagen). The resulting hybrid plasmid, pMsxB, was transformed into BLR(DE3)pLysS cells for controlled protein expression. Overexpression and optimal enzymatic activity of LpxM were achieved by growing the plasmid-containing cells in LB broth at 37 °C to A$_{600}$ of ∼0.6, followed by induction with 1 mM isopropylthio-β-D-galactoside and further incubation at 37 °C for an additional 4 h. Cell membranes were isolated and washed as described previously (36).

Coupled in Vitro Assays for LpxP and LpxM Activity—An assay system, pre-equilibrated at 12 °C without enzyme for 10 min, was set up so that the final concentrations of 50 mM HEPES, pH 7.5, 250 mM NaCl, 10 mM MgCl$_2$, 12.5 μM palmitoyl-ACP, 6 μM Kdo-4′-aza-p-L-lys, 80,000 cpm/mmol, and 10 μg/ml LpxP overproducing membranes from induced BLR(DE3)pLysS/pMKV2 cells in a final volume of 35 μl. Enzyme addition in 3.5 μl was used to start the reaction. The complete system was then incubated at 12 °C for 20 min. A 5-μl portion of the reaction mixture was spotted onto a Silica Gel 60 TLC plate and then the rest of the sample was split into two equal (15-μl) portions in microcentrifuge tubes and incubated at 30 °C. As indicated, membranes of the LpxM overproducer BLR(DE3)pLysS/p MsbB (0.5 l) were spotted on to TLC plates, which were developed in chloroform/methanol (4:1, v/v).

To assay LpxM and LpxP in the opposite order, the same reaction protocol was used except 12.5 μM myristoyl-ACP, instead of palmitoyl-ACP, was included in the initial assay mixture, and the 10 min pre-incubation in the absence of enzyme was at 30 °C, not 12 °C. As indicated, membranes from induced BLR(DE3)pLysS/pMsxB cells were then added to bring the final protein concentration to 0.5 μg/ml, and the reaction (35 μl) was allowed to proceed for 20 min at 30 °C. A 5-μl portion was removed and spotted onto a TLC plate. The reaction mixture was then split into two equal 15-μl portions, and the temperature was shifted to 12 °C, whereupon 12.5 μM palmitoyl-ACP (or water) and 10 μg/ml membranes of induced BLR(DE3)pLysS/pMKV2 cells were added to bring the volume of each tube to 20 μl, in analogy to the protocol described above.

RESULTS

Construction of Isogenic Mutants Lacking One or More of the Late Acyltransferases—The availability of the lpxP::kan mutant MKV11 (8), lacking the cold-induced palmitoleoyltransf erase (Table I), facilitated the construction of several new strains of E. coli with limited ability to synthesize acylacyl moieties. A P1$_{vir}$ bacteriophage lysate of MKV11 (a derivative of MC1061) was used to transduce the lpxP mutation into wild type W3110 and several existing lpxL and/or lpxM mutants (14, 18), already in the W3110 background, by selection for kanamycin resistance (Table I). Initially, all transduction mixtures were spread on 30 °C onto LB plates containing sodium citrate and kanamycin to select for the desired strains in which the lpxP::kan cassette had recombined into the chromosome. The lpxP mutation was first introduced into the wild type W3110 to generate the single step mutant MKV12 (Table I). The lpxM lpxP double mutant MKV14 (Table I) was likewise constructed using MLK1067 (18) as the recipient. However, attempts to construct lpxP lpxP double or lpxL lpxM lpxP triple mutants in this manner failed, as judged by the inability to recover kanamycin-resistant transductants in significant numbers on LB broth at 30 °C when MLK53 (lpxL) or MKL986 (lpxL lpxM) (14, 18) (Table I) were used as recipients. MLK53 and MKL986 themselves grew slightly less well than wild type on LB plates at 30 °C.

Another attempt was made to construct lpxP lpxP and lpxM lpxP mutants by P1$_{vir}$ transduction of the lpxP::kan cassette from MKV11 into MLK53 (lpxL) and MLK986 (lpxL lpxM) at 30 °C using minimal A plates with kanamycin for the selection. The rationale for this approach was the observation that MLK53 and MLK986 grew at precisely the same rate as wild type on minimal medium (14, 18) and were not temperature-sensitive. Reasonable numbers of kanamycin-resistant colonies were in fact observed after 36–48 h when the above transductions were carried out using minimal A plates. The desired mutants, MKV13 (lpxL lpxP) and MKV15 (lpxL lpxM lpxP) (Table I), were then re-purified as single colonies on minimal A plates at 30 °C. Like the parental strains MLK53 and MLK986, MKV13 and MKV15 were not temperature-sensitive on several types of minimal media. However, MKV13 and MKV15 were unable to form single colonies when streaked onto LB plates at both 30 and 42 °C, accounting for the initial failure to obtain these mutants.

Growth Rates of the Mutants in Minimal and Rich Media—To confirm the growth phenotypes seen on agar plates, all seven late acyltransferase mutants (Table I) and W3110 were grown in a rotary shaker at 225 rpm in Kozak minimal medium containing an NdeI restriction site at the 5′ end (5′-GCCGCCGCATGATGAGAAGCGAAAAAA-3′) and a BamHI site at the 3′ end (5′-GCCGCCGGATCCTTTGATGAGGATA-3′) were used. The PCR product was digested with NdeI and BamHI and ligated into the similarly digested vector, pET21a (Novagen). The resulting hybrid plasmid, pMsxB, was transformed into BLR(DE3)pLysS cells for controlled protein expression. Overexpression and optimal enzymatic activity of LpxM were achieved by growing the plasmid-containing cells in LB broth at 37 °C to A$_{600}$ of ∼0.6, followed by induction with 1 mM isopropylthio-β-D-galactoside and further incubation at 37 °C for an additional 4 h. Cell membranes were isolated and washed as described previously (36).

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FIG. 1. Growth of late acyltransferase mutants in minimal versus rich medium. A, all seven mutants grow at the same rate as wild type E. coli strain W3110 in minimal medium at 30 °C. B, after growing in minimal medium to early log phase at 30 °C, the cells were shifted to LB broth at 30 °C at time 0, and the cell density was monitored for several hours. MKV13 (lpxL lpxP) and MKV15 (lpxL lpxM lpxP) stopped growing after about 5 h in LB broth.

at 30 °C. The A_{600} was recorded at various times (Fig. 1A). As anticipated from their behavior on minimal medium plates, the growth rates of all the mutants were identical to wild type under these conditions.

To study the effects of nutrient broth on viability, the mutants were grown to early log phase in minimal medium. The cells were then collected by centrifugation, resuspended in the same volume of LB broth, and returned to rotary shaking at 225 rpm at 30 °C. As shown in Fig. 1B, MKL1067 (lpxM), MKV12 (lpxP), and MKV14 (lpxM lpxP) grew at the same rate as W3110 on LB broth (or about 4 times faster than on minimal medium). MKL53 (lpxL) and MLK986 (lpxL lpxM) showed slightly decreased growth rates compared with W3110 but reached the same final saturating density (Fig. 1B). In contrast, MKV13 (lpxL lpxP) and MKV15 (lpxL lpxM lpxP) stopped growing about 5 h after the shift from minimal medium to LB broth at 30 °C (Fig. 1B) and failed to reach the normal stationary phase density characteristic of wild type. The permissive effect of minimal medium on the growth of MKV13 and MKV15 (Fig. 1A) may reflect the general slowing of all other cellular metabolic processes so that these strains can export a sufficient amount of lipid A to maintain outer membrane assembly (see below).

Mass Spectrometry of Lipid A from lpxM and lpxM lpxP Mutants—The lipid A 1,4'-bisphosphate molecules synthesized by W3110 and each of the mutant strains listed in Table I were isolated by pH 4.5 hydrolysis at 100 °C of chloroform/methanol-extracted cells, followed by chromatography on DEAE-cellulose columns (12, 31). MALDI/TOF mass spectrometry in the negative mode was used to estimate the molecular weights of these substances. As shown in Fig. 2, both W3110 and MKV12 grown at 30 °C on minimal medium (as in Fig. 1A) synthesized the same, apparently wild type, lipid A 1,4'-bisphosphate species, as judged by the predominant molecular ions [M – H]^{-} at m/z 1797.8 and 1797.4, respectively (Fig. 3, structure A). The loss of the myristoyltransferase LpxM in MLK1067 or in MKV14 (lpxM lpxP) resulted in the synthesis at 30 °C of lipid A species characterized by molecular ions [M – H]^{-} at m/z 1587.1 or 1587.0, respectively (Fig. 2), which would arise from structure C (Fig. 3). The absence of any tetra-acylated lipid A species (Fig. 2) in either MLK1067 or MKV14 (m/z ~1405) shows that the lauroyltransferase LpxL is fully active in vivo despite the loss of LpxM and acylates all the available tetra-acylated precursors, Kdo2-lipid IV_{A} (see Fig. 1 of accompanying article (8)) in the absence of LpxM. The mass spectrometry (Fig. 2) furthermore reveals that LpxP is not induced at 30 °C as a compensatory mechanism when lpxM is inactivated, as judged by the absence of significant peaks above m/z 1587 in MLK1067.

Mass Spectrometry of Lipid A from lpxL, lpxL lpxP, and lpxL lpxM lpxP Mutants—The MALDI/TOF analyses of the lipid A molecules synthesized at 30 °C by various strains lacking lpxL (Fig. 4) are entirely consistent with the biosynthetic pathway (see Fig. 1 of accompanying article (8)) but reveal some additional subtleties. Although a significant amount of tetra-acylated lipid A accumulates in MLK53 (lpxL), as judged by the peak at m/z 1404.8 (Fig. 4), some penta-acylated and even some hexa-acylated lipid A molecules are still made, as demonstrated by the peaks at m/z 1615.0 and 1851.7 respectively. The peak at m/z 1851.7 (Fig. 4) arises from a lipid A molecule containing one secondary myristate and one secondary palmitoleate residue (Fig. 3, structure B), which is the same as the predominant form of lipid A seen in cold-shocked wild type cells (8). The peak at m/z 1615.0 is explained by the presence of a lipid A molecule containing only one secondary myristate chain (Fig. 3, structure E). This finding strongly suggests that LpxM can transfer some myristate directly to Kdo2-lipid IV_{A} in the absence of either laurate or palmitoleate but not at a rate that is sufficient to acylate all the available Kdo2-lipid IV_{A}. Previous in vitro studies have in fact shown that Kdo2-(lauroyl)-lipid IV_{A} is the preferred substrate for LpxM but that Kdo2-lipid IV_{A} can also be utilized at about 1% the rate (6). The alternative possibility that LpxM attaches a secondary myristate chain at the 2'-position is extremely unlikely, because the resulting molecule would be an excellent substrate for further LpxM-catalyzed acylation of the 3'-secondary position, resulting in a hexa-acylated product with a molecular weight of 1826.4.

The idea that LpxM can function (albeit slowly) in the absence of LpxL is substantiated by the lipid A analysis of MKV13 (lpxL lpxP) (Fig. 4), in which about one-third of the
lipid A appears to be penta-acylated with myristate as the sole secondary acyl chain at position 3', as demonstrated by the peak at m/z 1615.2. The rest is tetra-acylated, as shown by the large signal at m/z 1404.7. Finally, the absence in MKV13 of the peak at m/z 1851.7 seen in MLK53 (Fig. 4) is consistent with the absence of LpxP in MKV13 and confirms that lpxP is partially induced at 30 °C in MLK53 (lpxL) as a compensatory mechanism, in contrast to the situation in MLK1067 (lpxM) or wild type cells (Fig. 2).

The MALDI/TOF analysis of the lipid A isolated from MLK986 (lpxL lpxM) (Fig. 4) reveals that, in addition to tetra-acylated lipid A (m/z 1404.8), there is a substantial peak at m/z 1641.2, which is interpreted as the [M – H]− ion of a lipid A molecule containing a single secondary palmitoleate residue (Fig. 3, structure D). As in MLK53, it appears that the inactivation of the lpxL gene in MLK986 results in the compensatory induction of LpxP in cells grown at 30 °C, whereas in isogenic strains containing a functional lpxL gene no evidence for lpxP activation is seen at 30 °C (Fig. 2).

Mass Spectrometry of Lipid A from the lpxL lpxM lpxP Triple Mutant—The triple mutant strain MKV15 (Fig. 4), constructed by transduction of the lpxP::kan allele of MKV11 into MLK986 (lpxL lpxM), produced only a tetra-acylated lipid A molecule (Fig. 3, structure F), as judged by the predominant peak at m/z of 1404.7. There was no evidence that the palmitoyltransferase activity of PagP (see Fig. 1 of, accompanying article (8) and Ref. 11) was induced in the triple mutant, because no signal was observed near m/z 1643. Furthermore, no other lipases or
novel alternative acyltransferases were activated to compensate for the loss of \( \text{lpxL}, \text{lpxM}, \text{and lpxP} \).

**Radiochemical Profiling of the Lipid A Species Produced by the Late Acyltransferase Mutants**—An independent method was used to estimate the relative amounts of the lipid A species produced by each of the strains in a more quantitative manner than afforded by mass spectrometry. The cells were labeled for several generations with \( ^{32}\text{P} \) during exponential growth at 30 °C in G56 low phosphate minimal medium (27), and all the \( ^{32}\text{P} \)-labeled lipid A species were extracted on a small scale (following release from cells by pH 4.5 hydrolysis at 100 °C) without fractionation on DEAE-cellulose columns (12, 37). The \( ^{32}\text{P} \)-labeled lipid A preparations from each of the strains were spotted directly onto a thin layer chromatography plate, which was developed in the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v) and analyzed with a Phosphor-Imager.

As expected, wild type W3110 (Fig. 5, lane 1) contained primarily hexa-acylated lipid A (Fig. 3, structure A) and a small amount of penta-acylated lipid A (Fig. 3, structure C). MKV12 (\( \text{lpxP} \)) grown at 30 °C exhibited a profile identical to W3110 (Fig. 5, lane 2). Both MLK1067 (\( \text{lpxM} \)) (Fig. 5, lane 5) and MKV14 (\( \text{lpxM lpxP} \)) (Fig. 5, lane 6) grown at 30 °C contained only penta-acylated lipid A (Fig. 3, structure C), consistent with the mass spectrometry (Fig. 2). However, the various lipid A 1-pyrophosphates (12, 37) noted in Fig. 5 were not seen during mass spectrometry (Fig. 2) because they, together with phospholipid and lipid A degradation products (Fig. 5), were removed by chromatography on DEAE-cellulose prior to mass spectrometry (12, 37).

The reason for the presence of a small amount penta-acylated lipid A in the \( ^{32}\text{P} \)-lipid A profiles (Fig. 5) of W3110 and MKV12 is unclear, because this material was absent in the MALDI/TOF analysis (Fig. 2). One possible explanation
for this anomaly is that two minimal media, differing greatly in their phosphate concentrations, were employed for the mass spectrometry versus the $^{32}$P labeling (see “Experimental Procedures”).

MLK53 (lpxL) (Fig. 5, lane 3) contains $^{32}$P-labeled hexa-acylated, penta-acylated, and tetra-acylated lipid A species, consistent with mass spectrometry (Fig. 4). The hexa-acylated lipid A of MLK53 migrates slightly faster than that of W3110, presumably because the palmitoleate substituent (Fig. 3, structure B) is more hydrophobic than laurate (Fig. 3, structure A).

MKV13 (lpxL lpxP) (Fig. 5, lane 4) synthesizes a mixture of penta-acylated (Fig. 3, structure E) and tetra-acylated lipid A molecules (Fig. 3, structure F), also seen by mass spectrometry (Fig. 4). MLK986 (lpxL lpxM) (Fig. 5, lane 7) shows a $^{32}$P-lipid A profile similar to that of MKV13 (Fig. 5, lane 4), but in the case of MLK986 the penta-acylated lipid A species is structure D rather than structure E (Fig. 3). Finally, the $^{32}$P-labeling experiment confirms that MKV15 (lpxL lpxM lpxP) (Fig. 5, lane 8) synthesizes only a tetra-acylated lipid A species.

Transport of Tetra-acylated Lipid A to the Outer Membrane in MKV15—As discussed previously (17, 22), the acylation state of lipid A may affect the ability of the E. coli lipid transport machinery to move LPS from its site of biosynthesis on the inner membrane to the outer membrane. To study the consequences of eliminating all secondary acyl chains on lipid A transport, wild type W3110 and the triple mutant MKV15 were labeled with $^{32}$P, for 10 min during exponential growth at 30 °C in low phosphate minimal medium. The cells were rapidly...
harvested and broken by sonic irradiation. Inner and outer membranes were collected and separated on an isopycnic sucrose gradient (data not shown) (36). The peak fractions containing inner and outer membranes were pooled separately, and the molar ratio of total lipid A to total glycerophospholipids was determined by a combination of thin layer chromatography and PhosphorImager analysis. As shown in Fig. 6, neither the membrane localization nor the relative quantity of the glycerophospholipids was significantly different in the triple mutant versus the wild type during a 10-min labeling with $^{32}$Pi. In contrast, the lipid A from the triple mutant was largely localized in the inner membrane following a 10-min pulse labeling, although most of the lipid A was transported to the outer membrane during this period in wild type cells. The molar ratios of lipid A to glycerophospholipids (Table II) in isolated membranes and whole cells confirmed these results. The apparent delay in lipid A export was accentuated when exponentially growing mutant cells were shifted from minimal medium to LB broth for a short time (as in Fig. 1B) and then labeled with $^{32}$Pi for 10 min (Table II). These findings may explain why MKV15 and MKV13 are unable to grow on LB broth at 30°C (Fig. 1B). Antibiotic Hypersensitivity of Late Acyltransferase Mutants—Several different antibiotics were tested to evaluate the effects of insertions in late acyltransferase genes on outer membrane function (Table III). As expected, the lpxP mutant MKV12 and the lpxM lpxP double mutant MKV14 were no more sensitive to antibiotics than their respective parental strains, W3110 and MLK1067, when grown on minimal medium at 30°C. Surprisingly, the lpxL lpxP mutant MKV13 was nearly as sensitive to the antibiotics as its parental lpxL strain MLK53, even though MKV13 contains no hexa-acylated lipid A (Figs. 4 and 5). The same was true of the triple mutant MKV15 versus its parental strain MLK986 (lpxL lpxM), which differed only slightly in their sensitivity to one of the antibiotics, rifampicin, despite the fact that MLK986 contains penta-acylated lipid A and MKV15 does not. The overall conclusion is that loss of lpxL by itself causes the most dramatic increase in antibiotic sensitivity in this series, with lpxM contributing only slightly to outer membrane function, and lpxP contributing very little in cells grown at 30°C. In no instance is the antibiotic hypersensitivity of late acyltransferase mutants as striking as that observed for lpxA, lpxC, or lpxD point mutants, grown under semi-permissive conditions with about a 30% reduction of their total lipid A content (38, 39).
E. coli Mutant Lacking the lpxL, lpxM, and lpxP Genes

DISCUSSION

We have constructed a complete set of E. coli mutants containing antibiotic resistance cassettes in the genes encoding the three ACP-dependent late acyltransferases of lipid A biosynthesis (5–7, 16) (see Fig. 1 of accompanying article (8)). These mutants produce lipid A species with altered secondary acylation patterns, as determined by MALDI/TOF mass spectrometry (Figs. 2–4) and \(^{32}\)P labeling (Fig. 5). An E. coli triple mutant (MKV15) with no secondary acyl chains was constructed for the first time and found to be viable on minimal medium (Fig. 1A) but not on LB broth at 30 °C (Fig. 1B) or above. A plausible explanation for this phenomenon is that the rate of LPS export from the inner membrane (the site of lipid A production) to the outer membrane is slowed because of the absence of the secondary acyl chains (Fig. 6) (17), especially when the mutant is shifted to LB broth (Fig. 1B and Table II). Inhibition of LPS export might be more detrimental to cells in rich media because other processes, like DNA replication, protein translation, and phospholipid biosynthesis, proceed at a faster rate. In minimal media, however, these processes would be slowed, providing enough time for balanced outer membrane assembly despite the slow export of tetra-acylated lipid A.

Tetra-acylated lipid A may not be efficiently exported because the transport machinery may require a certain degree of hydrophobicity or an ideal shape, perhaps requiring the presence of at least one secondary acyl chain on lipid A. Diverse Gram-negative bacteria all synthesize lipid A species with at least one or two secondary acyl chains (1, 3, 40). Gram-negative bacteria all synthesize lipid A species with at least one secondary acyl chain on lipid A. Diverse Gram-negative bacteria all synthesize lipid A species with at least one or two secondary acyl chains (1, 3, 40). Diverse Gram-negative bacteria all synthesize lipid A species with at least one or two secondary acyl chains (1, 3, 40).

However, the extreme antibiotic hypersensitivity associated with mutations in the early steps of the lipid A pathway (43) is not seen with any of the late acyltransferase mutants.

As noted above, the triple mutant is very susceptible to spontaneous lysis when cells are collected by centrifugation. The significance of this observation is unclear, but it suggests that peptidoglycan synthesis or remodeling might be compromised. Direct measurements of peptidoglycan synthesis and cross-linking, in conjunction with electron microscopy, should shed some light on this phenomenon. Karow et al. (14, 18) noted that lipid A and lipid A lipid A mutants displayed unusual bulges under the phase contrast microscope, consistent with the idea of compromised peptidoglycan integrity.

The MALDI/TOF data (Figs. 2 and 4) and the \(^{32}\)P-lipid A profiles (Fig. 5) provide new insights into the functioning of the late acyltransferases in living cells. Very little of the penta-acylated lipid A that accumulates in MLK53 (Figs. 4 and 5) contains palmitoleate as its secondary acyl chain (Fig. 3, structure D). Instead, the penta-acylated material found in MLK53 contains myristate as its sole secondary acyl chain (Fig. 3, structure E). This unexpected finding has several important implications. 1) Given that the hexa-acylated lipid A present in MLK53 contains both palmitoleate and myristate, any Kdo₂-lipid A that is first acylated by the palmitoleoyltransferase must be very rapidly myristoylated by LpxM. 2) The myristoyltransferase must be capable of acylating Kdo₂-lipid A directly at position 3′ in the absence of a secondary acyl chain at position 2′, as shown by the analysis of the lipid A species in both MLK53 and MKV13 (Fig. 4). However, secondary acylation at position 3′ is not very rapid when a 2′ secondary acyl chain is missing, as indicated by the substantial amount of residual tetra-acylated lipid A in both MLK53 and MKV13. 3) The presence of relatively more palmitoleate containing lipid A species in MLK986 versus MLK53 (Figs. 4 and 5) suggests that palmitoleate is not incorporated efficiently once myristate is attached at position 3′. An enzymatic scheme that accounts for these findings is held some light on this phenomenon. Karow et al. (14, 18) noted that lipid A and lipid A lipid A mutants displayed unusual bulges under the phase contrast microscope, consistent with the idea of compromised peptidoglycan integrity.

To confirm that palmitoleate is not incorporated efficiently after the secondary 3′-myristate chain is in place (Fig. 7), an in vitro lipid A acylation system (4–6) was set up using membranes from strains that overexpress lipid A or lipid A behind the T7lac promoter. As shown in Fig. 8, Kdo₂-4-\(^{32}\)P-lipid IVₐ that is acylated with palmitoleate by incubation with 10 µg/ml LpxP-containing membranes in the presence of palmitoleoyl-ACP at 30 °C (lanes 6–8). Omission of myristoyl-ACP prevents the formation of hexa-acylated lipid A (lanes 9–11), demonstrating that LpxP cannot catalyze a second acylation and that LpxM cannot utilize palmitoleoyl-ACP. However, when the LpxM membranes are used first at the same concentration (0.5 µg/ml) to acylate some of the Kdo₂-4-\(^{32}\)P-lipid IVₐ with myristate.

TABLE III

| Strain    | Genotype | Rifampicin (µg/ml) | Erythromycin (µg/ml) | Vancomycin (µg/ml) | Bacitracin (µg/ml) | Fusidic acid (µg/ml) |
|-----------|----------|-------------------|---------------------|-------------------|-------------------|---------------------|
| W3110     | Wild type| 10                | 500                 | 500               | >500              | >1000               |
| MKV12     | lpxP     | 10                | 500                 | 500               | >500              | >1000               |
| MLK53     | lpxL     | 1                 | 50                  | 50                | 500               | 1000                |
| MKV19     | lpxL lpxP| 1                 | 50                  | 50                | 500               | 1000                |
| MLK1067   | lpxM     | 5                 | 500                 | 500               | >500              | >1000               |
| MKV14     | lpxM lpxP| 5                 | 500                 | 500               | >500              | >1000               |
| MLK986    | lpxL lpxM| 1                 | 50                  | 50                | 500               | 1000                |
| MKV15     | lpxL lpxM lpxP| 0.5 | 50 | 50 | 100 | 1000 |
(lane 12), only as much myristate transfer is seen after 20 min (lane 12) as is seen with Kdo₂-4⁻³²P-(C₁₆:1)-lipid IVA in 1 min (lane 6). Obviously, LpxM prefers the penta-acylated substrate Kdo₂-4⁻³²P-(C₁₆:1)-lipid IVA over Kdo₂-4⁻³²P-lipid IVA by at least an order of magnitude.

In an additional experiment (Fig. 8, lanes 13–15), the LpxM reaction mixture shown in lane 12 was further incubated for an additional 15 min with added LpxP membranes and palmitoleoyl-ACP. The temperature was shifted to 12 °C to avoid LpxP inactivation, but at 12 °C LpxM would still be functional. As shown in lanes 13–15, the amount of Kdo₂-4⁻³²P-(C₁₄:0)-lipid IVA stays relatively constant to that seen in lane 12, as judged by densitometry. However, incorporation of palmitoleate into Kdo₂-4⁻³²P-lipid IVA in preference to Kdo₂-4⁻³²P-(C₁₆:1)-lipid IVA by LpxM by at least an order of magnitude.

The induction of the palmitoleoyltransferase at 30 °C, seen in MLK53 and MLK986, is somewhat surprising, because this activity is normally absent in wild type cells at 30 °C (7). Up-regulation at 30 °C of a protein that is normally cold shock-induced may occur via a regulatory circuit that is distinct from what goes on in wild type cells. This process might involve an alternate promoter that is activated at 30 °C in the absence of LpxL, production of an RNA-binding protein to protect lpxP mRNA from degradation, or formation of a protein partner to keep LpxP properly folded at 30 °C. Detailed studies into the regulation of lpxP expression in wild type and mutant cells are currently underway in our laboratory.

The E. coli mutants described in the present study may offer some new possibilities for the development of vaccines and may also serve as improved sources of novel lipid A molecules. Tetra-acylated lipid IVA, which is easy to isolate from MKV15, is an antagonist of LPS-induced activation of human monocytes. Lipid A from the photosynthetic bacterium Rhodobacter sphaeroides (44, 45) is likewise an antagonist of LPS-induced inflammation in many animal systems. The structure of R. sphaeroides lipid A contains a mono-unsaturated 14-carbon secondary acyl chain at the 2'-position (44), not unlike the palmitoleate seen in lipid A species D (Fig. 3) that accumulates in MLK986. However, the biological properties of these new lipid A variants have not yet been explored.

Some of the late acyltransferase mutants may be sufficiently

**Fig. 7.** Enzymatic scheme for the attachment of secondary acyl chains to lipid A precursors at 30 °C in an E. coli mutant lacking lpxL. Neither LpxM nor LpxP are very active in living cells at 30 °C when LpxL is inactivated, accounting for the presence of considerable amounts of tetra-acylated lipid A in MLK53 (Fig. 4). LpxM prefers a penta-acylated substrate containing a secondary acyl chain at position 2', but presumably not very much LpxP is induced in these mutants. Penta-acylated lipid A molecules containing a secondary myristate chain at position 3' are also poor substrates for LpxP.
isoate second site suppressors of MKV15 that do. Like MKV15, these second site suppressor-containing strains synthesize only tetra-acylated lipid A. The identification of the second site suppressor mutation(s) that enables the triple mutant to grow on rich medium might shed further light on the function of the secondary acyl chains present on lipid A and on the mechanism of lipid A export to the outer membrane.

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