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Function of *Escherichia coli* MsbA, an Essential ABC Family Transporter, in Lipid A and Phospholipid Biosynthesis*

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The *Escherichia coli msbA* gene, first identified as a multicopy suppressor of *htrB* mutations, has been proposed to transport nascent core-lipid A molecules across the inner membrane (Polissi, A., and Georgopoulos, C. (1996) *Mol. Microbiol.* 20, 1221–1233). *msbA* is an essential *E. coli* gene with high sequence similarity to mammalian Mdr proteins and certain types of bacterial ABC transporters. *htrB* is required for growth above 32 °C and encodes the lauroyltransferase that acts after Kdo addition during lipid A biosynthesis (Clementz, T., Bednarski, J., and Raetz, C. R. H. (1996) *J. Biol. Chem.* 271, 12095–12102). By using a quantitative new $^{32}P_i$ labeling technique, we demonstrate that hexa-acylated species of lipid A predominate in the outer membranes of wild type *E. coli* labeled for several generations at 42 °C. In contrast, in *htrB* mutants shifted to 42 °C for 3 h, tetra-acylated lipid A species and glycerophospholipids accumulate in the inner membrane. Extra copies of the cloned *msbA* gene restore the ability of *htrB* mutants to grow at 42 °C, but they do not increase the extent of lipid A acylation. However, a significant fraction of the tetra-acylated lipid A species that accumulate in *htrB* mutants are transported to the outer membrane in the presence of extra copies of *msbA*. *E. coli* strains in which msbA synthesis is selectively shut off at 42 °C accumulate hexa-acylated lipid A and glycerophospholipids in their inner membranes. Our results support the view that MsbA plays a role in lipid A and possibly glycerophospholipid transport. The tetra-acylated lipid A precursors that accumulate in *htrB* mutants may not be transported as efficiently by MsbA as are penta- or hexa-acylated lipid A species.

Lipopolysaccharide (LPS)[1] is a major component of the outer leaflet of the outer membranes of Gram-negative bacteria (1–5). The hydrophobic membrane anchor of LPS is termed lipid A (2, 3, 6, 7). It is glycosylated with a non-repeating oligosaccharide, known as the core (2–4). In most clinical isolates, the distal end of the core is further derivatized with O-antigen, a large polysaccharide made up of 1–40 distinct oligosaccharide repeats (2–4, 8).

Many of the key enzymatic reactions involved in *Escherichia coli* LPS biosynthesis are known (2, 4). Lipid A with its attached core (designated core-lipid A) and the O-antigen are synthesized as separate units (2, 4). The important studies of Osborn and co-workers (9–14) suggest that O-antigen is ligated to nascent core-lipid A on the periplasmic surface of the inner membrane (Fig. 1). The core-lipid A domain is made in association with the inner surface of the inner membrane (2, 4). It must therefore be flipped across the inner membrane prior to O-antigen ligation. O-antigen attachment is not required for core-lipid A transit to the outer membrane, given that LPS consisting only of Kdo_2-lipid A (Fig. 2) supports the growth of *E. coli* (2, 4) and is translocated efficiently (7, 10).

Elucidation of the molecular details of LPS transit from the cytoplasm to the outer membrane (7, 10) remains one of the most intriguing, unsolved problems of LPS biochemistry (Fig. 1). A possible clue to the mechanism of core-lipid A transport within the inner membrane has emerged from the recent discovery that HtrB functions as the lauroyltransferase that acts immediately after Kdo addition during lipid A biosynthesis (Fig. 2) (15, 16). Cells harboring *htrB* mutations fail to grow above 32 °C (17), display unusual surface bulging (17), overproduce glycerophospholipids at 42 °C (17, 18), and accumulate under-acylated species of lipid A (19).

htrB insertion mutations were first isolated by Karow and Georgopoulos (17) during a search for new *E. coli* heat shock genes. Two multicopy suppressors of *htrB* mutations (designated *msbA* and *msbB*) were also reported (20, 21). The *msbB* gene displays sequence homology to *htrB* (20, 22) and encodes a distinct acyltransferase (16, 19) (Fig. 2) that can substitute for *htrB* at 42 °C, when overexpressed on high copy plasmids. The *msbA* gene shows no sequence relationship to *htrB* (21). Instead, *msbA* encodes an ABC family transporter with high amino acid sequence similarity to mammalian Mdr proteins (21). *msbA* is an essential gene (21), whereas *msbB* is not (20, 23). When introduced on low copy plasmids, *msbA* complements most of the *htrB* mutant phenotypes, such as the morphological alterations, the overproduction of phospholipids, and the inability to grow above 32 °C (21).

Polissi and Georgopoulos (24) have recently shown that MsbA is an inner membrane, ATP-binding protein, as predicted from its sequence. When *msbA* and an essential downstream gene, *orfE*, are both inactivated because of an insertional mutation in *msbA* in a strain with a temperature-sensitive replication plasmid expressing both MsbA and OrfE, LPS accumulates in the inner membrane at 42 °C (24). Similar findings have recently been reported (25) using the *msbA*(*valA*) and *orfE*(*valB*) genes of *Francisella novicida*. Moreover, LPS accumulates in the inner membranes of *htrB*-deficient mutants (24). Introduction of *msbA* and *orfE* on low copy plasmids partially restores translocation of LPS to the outer membrane

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[1] The abbreviations used are: LPS, lipopolysaccharide; PCR, polymerase chain reaction; Kdo, 3-deoxy-D-*manno*-octulosonic acid.

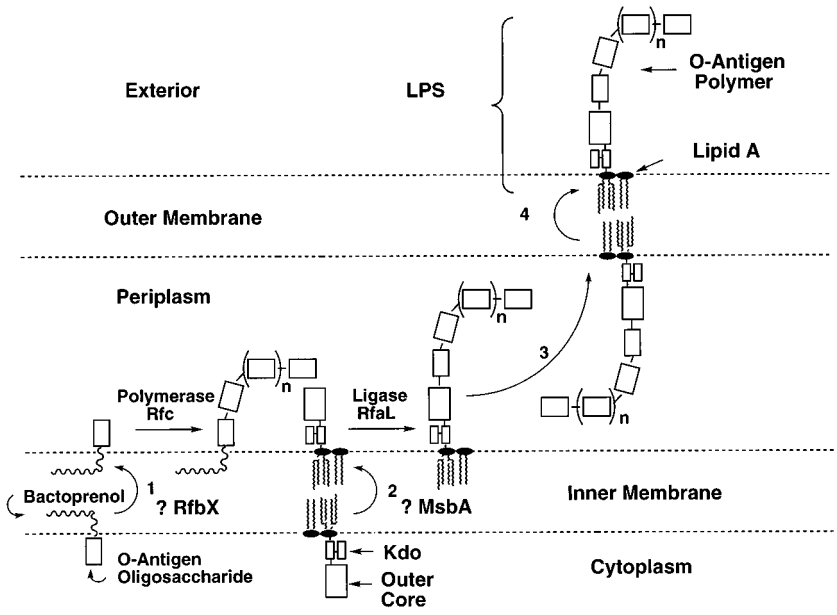

FIG. 1. **A possible role for MsbA in the transport of core-lipid A molecules across the inner membrane of *E. coli*.** The proposed function of MsbA as an inner membrane "flippase" for nascent core-lipid A molecules is indicated. Nothing is known about possible protein factors that might assist in the translocation of completed LPS and core-lipid A molecules across the periplasm or in their further assembly within the outer membrane. However, transport of core-lipid A molecules to the outer membrane can occur independently of ligation to O-antigen. Alternative transport models are discussed elsewhere (7, 10) but seem less likely, given the experimental evidence for periplasmic O-antigen intermediates (9, 12). Recently, RfbX(Wzx) has been proposed as a possible O-antigen flippase (51). Quantitative *in vitro* assays for O-antigen polymerization and ligation have not been described (2). The accumulatiom of phospholipids in the inner membranes of *msbA* mutants suggests that MsbA might also transport phospholipids.

at 42 °C in *htrB* mutants (24). Taken together, these findings suggest that the HtrB-catalyzed transfer of laurate to lipid A (Fig. 2) may be necessary for efficient core-lipid A transport and that MsbA and/or OrfE may be components of the transport machinery.

Garrett *et al*. (26) have recently discovered that *orfE* (now designated *lpxK*) encodes the 4′-kinase of the lipid A pathway (53), a key enzyme that functions several steps before HtrB (Fig. 2). We have therefore re-evaluated the contribution of *orfE*/*lpxK* and *msbA* to the suppression of the *htrB* phenotype. We now show that the rescue of *htrB* mutants by overexpression of *msbA* is not accompanied by an increase in the extent of lipid A acylation at 42 °C. By using a quantitative new $^{32}P_i$ labeling assay, we demonstrate that *htrB*-deficient cells accumulate tetra-acylated lipid A molecular species and glycerophospholipids within their inner membranes at 42 °C, whereas *msbA*-deficient strains accumulate hexa-acylated lipid A and glycerophospholipids in their inner membranes, even when extra copies of *orfE*/*lpxK* are provided in *trans*. Our data strengthen the view that MsbA is involved in the transport of newly synthesized core-lipid A molecules across the inner membrane (24, 25) and suggest that MsbA may also transport glycerophospholipids.

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ and [1-^{14}C]dipalmitoyl-phosphatidylcholine were purchased from NEN Life Science Products. Glass-backed Silica Gel 60 thin layer chromatography plates (0.25 mm) were from Merck, Germany. Restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, and *Sac*II were purchased from New England Biolabs, and the Wizard *Plus* Miniprep DNA purification system was from Promega. Triton X-100 was obtained from Pierce and NADH from Sigma. Pyridine, methanol, and 88% formic acid were obtained from Mallinckrodt. Chloroform was purchased from EM Science. Chemicals of analytical grade were used throughout.

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are described in Table I. Cells were generally grown at 30 or 42 °C in LB broth, consisting of 10 g of NaCl, 5 g of yeast extract, and 10 g of tryptone per liter (27). Antibiotics were added when necessary at final concentrations of 50 μg/ml for ampicillin, 12 μg/ml for tetracycline, 10 μg/ml for chloramphenicol, 50 μg/ml for spectinomycin.

Recombinant DNA Techniques and Construction of pEH2—Plasmid DNAs were isolated by using the Wizard Plus Miniprep DNA purification system (Promega). Restriction endonucleases, T4 DNA ligase, and shrimp alkaline phosphatase were used according to the manufacturers' specifications. Other recombinant DNA techniques were performed as described by Ausubel *et al.* (28).

The pEH2 subclone containing the *orfE*/*lpxK* gene was made by digesting plasmid pGAP14 DNA with *Eco*RI and *Hin*dIII (24) and ligating the 1.9-kilobase pair fragment derived from pGAP14 with *Eco*RI and *Hin*dIII-digested pUC18 vector DNA. The ability of pEH2 to direct the overexpression (~5-fold) of the lipid A 4′-kinase was confirmed by assaying extracts of cells (26) containing pEH2 compared with vector controls.

Construction of pKW2 Bearing the msbA Gene—Genomic *E. coli* DNA was isolated from strain W3110 according to Ausubel *et al*. (28). After the chromosomal DNA was isolated, the *msbA* gene was amplified by the polymerase chain reaction (PCR). The primer sequences used were as follows: the forward primer, 5′ GCG CTC TAT CTC GCT CAA GGC TGG CGG ATT AGC AGC CTC AGG GAG C 3′ and the reverse primer, 5′ GCG CGC ATC GAT GGC CAT ACA ACC AGG AGA GTG GCA GCA ATA GCC GCC. The PCR reaction contained 100 ng of W3110 chromosomal DNA template, 0.2 μg of each primer, 10 mM Tris-HCl, pH 9.2, 1.5 mM $MgCl_2$, 75 mM KCl, 200 μM each dNTP, and 1 unit of *Taq* polymerase (Stratagene). The mixture was subjected to 5 min of denaturation at 94 °C, then 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and ended with a 7-min extension at 72 °C in a Perkin-Elmer GeneAmp PCR system 2400. The PCR product was then ligated into the TA cloning vector and transformed into the appropriate competent cells (Invitrogen Corp.) according to the manufacturer's instructions. Plasmid bearing colonies were selected by growth on LB agar plates containing 50 μg/ml ampicillin and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside at 37 °C. Plasmid was isolated from positive (white) colonies, and the presence of the insert was confirmed by digestion with *Hin*dIII and *Eco*RV. Due to the reported sensitivity of the MLK53 cells to the presence of the *msbA* on high-copy vectors (21), we chose to move the insert into the low-copy vector, pACYC184 (New England Biolabs, Inc.). The TA plasmid bearing the *msbA* gene (pKW1) was digested with *Hin*dIII and *Eco*RV. The reaction was subjected to gel electrophoresis, and the insert fragment was purified using the GeneClean system (Bio 101). Simultaneously, the plasmid pACYC184 was digested with *Hin*dIII and *Eco*RV, phosphatase- treated, and purified as described above for the insert. The digested pACYC184 and the insert were ligated. The ligation reactions were then transformed into SureCells (Stratagene), and plasmid containing cells were selected by growth on LB agar containing 30 μg/ml chloramphenicol. The plasmid was isolated from chloramphenicol-resistant cells, and the presence of the insert was confirmed by digestion with *Hin*dIII and *Eco*RV. The pACYC184 containing *msbA* was named pKW2.

The DNA sequence of the insert was confirmed by automated fluorescent sequencing at the Duke University DNA Analysis Facility, Durham, NC. The sequencing of pKW2 revealed that the plasmid contained the predicted junctions between pACYC184 (*Hin*dIII and *Eco*RV) and the expected TA sequence. However, the first 17 nucleotides of the forward primer, 5′ **GCG CTC TAT CTC GCT CAA G**GC TGG CGG ATT AGC AGC CTC AGG GAG C 3′ (shown in bold) were not present.

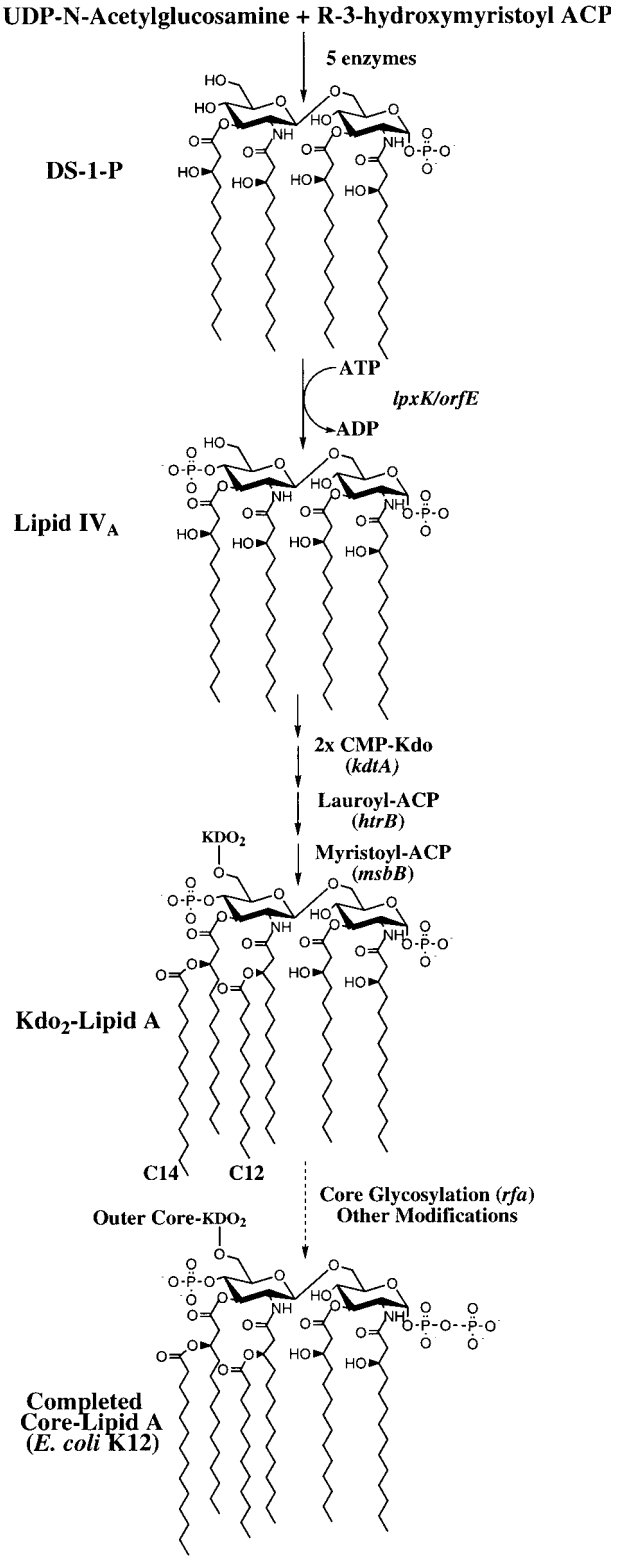

FIG. 2. **Functions of *orfE/lpxK*, *htrB*, and *msbB* in the biosynthesis of Kdo$_2$-lipid A in *E. coli*.** Five enzymes are required for the synthesis of the intermediate designated DS-1-P, the physiological substrate for the 4′-kinase (2). Structural genes encoding each of the indicated enzymes are in *italics*. The gene for the 4′-kinase has recently been identified as *orfE* (the downstream gene co-transcribed with *msbA*) (26). *orfE* is now designated *lpxK*. Following phosphorylation by the 4′-kinase, the Kdo transferase (encoded by *kdtA*) adds two Kdo residues to lipid IV$_A$ to form Kdo$_2$-lipid IV$_A$ (41, 52). The Kdo-dependent acyltransferases, HtrB and MsbB, then add laurate and myristate, respectively, to form Kdo$_2$-lipid A (16, 19). Kdo$_2$-lipid A (with only five acyl chains) is sufficient to support the growth of *E. coli*. However, overexpression of *msbA* can suppress the temperature-sensitive phenotype of strains lacking *htrB* (21) and allows growth with only four acyl chains on lipid A. At 30 °C, other acyltransferases, such as Ddg (37) (not shown), may substitute for HtrB. Finally, core sugars and other modifying groups are incorporated, including the additional phosphate residue present on a portion of the lipid A moieties of *E. coli* K-12, designated the trisphosphate species (*bottom structure, dashed bond*).

TABLE I
Strains and plasmids used in this study

Strain/plasmid	Relevant genotype	Source or refer.
W3110	Wild type, F$^-$, lambda$^-$	*E. coli* genetic stock center, Yale University
MLK53	W3110 *htrB1*::Tn10	16, 17
MLK1067	W3110 *msbB1*::ΩCam	19, 20
AP70	W3110 *msbA*::ΩCam *recA56 srl*::Tc pGAP10 (*msbA*$^+$/*orfE*$^+$)	24
AP191	W3110 *msb*A::ΩCam *recA56 srl*::Tc pGAP14 (*msbA*$^+$/*orfE*$^+$) (TS replicon)	24
pKW2	pACYC184 containing *msbA*$^+$	This study
pEH2	pUC18 containing *Eco*RI-*Hin*dIII *orfE* fragment from pGAP14	This study

The absence of this portion of the primer sequence did not disrupt the coding sequence of *msbA*, since the primer was designed to a segment of DNA 92 nucleotides upstream of the start codon of *msbA*. The *msbA* coding sequence determined for pKW2 (data not shown) exactly matched the published *msbA* sequence (21).

Finally, the *htrB* mutant MLK53 was transformed with pKW2 at 30 °C, selecting for tetracycline and chloramphenicol resistance. MLK53 is temperature-sensitive for growth at 44 °C. When competent MLK53 cells were transformed at 30 °C with pKW2, the temperature sensitivity was suppressed in all the transformants. In other experiments, MLK53 was transformed with pKW2 and subjected immediately to selection at 44 °C. The frequency of transformants was the same as observed at 30 °C.

Growth of $^{32}P_i$-Labeled Cells—Cells were grown and labeled using the methods described previously (19, 29). In general, labeling of the glycerophospholipids and lipid A was achieved by dilution of overnight cultures with 125 ml of pre-warmed fresh LB broth containing appropriate antibiotics to yield A_{550} = 0.05. After addition of 4 μCi/ml $^{32}P_i$, non-temperature-sensitive and wild type cells were grown at 30 or 42 °C (as indicated) until A_{550} = 0.6. All temperature-sensitive mutants were labeled for 3 h after a shift to 42 °C. At the time of harvest, their A_{550} was 0.4–0.6.

Membrane Fractionation—Inner and outer membranes were separated by isopycnic sucrose gradient centrifugation following the procedure of Guy-Caffey *et al.* (30) with several modifications. Disruption of the lysozyme- and EDTA-treated spheroplasts was performed using a Branson sonifier (model 250) equipped with a microtip (31). Sonic disruption was performed while the sample was immersed in an ice-water bath. One-minute intervals were allowed for cooling between each 30-s period of sonic oscillation (80 watts of power output). The whole particulate fraction was isolated on a sucrose cushion and washed once. Inner and outer membranes (recovered from 125 ml of cells and containing about 2 × 10^6 cpm of ^{32}P) in 1.5 ml of 10 mM Tris acetate buffer, pH 7.8, with 0.5 mM EDTA were separated on a sucrose gradient prepared by layering 0.4 ml of 60% sucrose, 0.9 ml of 55% sucrose, 2.2 ml of 50% sucrose, 2.2 ml of 45% sucrose, 2.2 ml of 40% sucrose, 2.2 ml of 35% sucrose, and 0.4 ml of 30% sucrose (30). The gradient was buffered with 10 mM Tris acetate, pH 7.8, containing 0.5 mM EDTA. The separated membranes were collected in ~0.4-ml fractions by piercing the bottom of the centrifuge tube. Radioactivity in each fraction was measured with a scintillation counter.

Enzymatic Assays—Phospholipase A activity (an outer membrane marker) was determined as described (32) with minor modifications. The reaction mixture, prepared in a 0.6-ml plastic microcentrifuge tube,

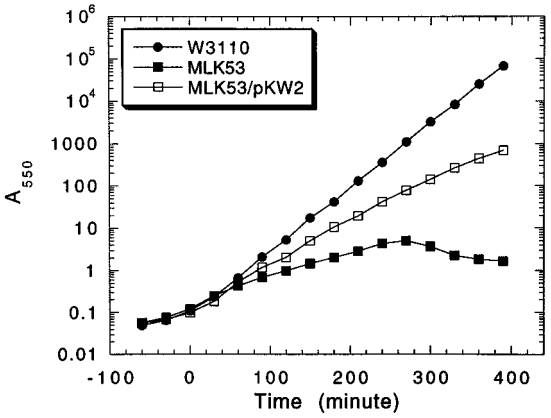

FIG. 3. **Temperature-sensitive growth of an *E. coli* mutant lacking *htrB* and its suppression by extra copies of *msbA*.** Strains were grown overnight at 30 °C on LB broth in the presence of appropriate antibiotics. The cultures were diluted into pre-warmed fresh medium and were allowed to grow at 30 °C until A_{550} reached 0.1. The growth temperature was then shifted to 42 °C (time 0). Cultures were intermittently diluted to maintain A_{550} below 0.4. The absorbance shown in the figure represents the cumulative growth yield and not the actual A_{550} of the culture.

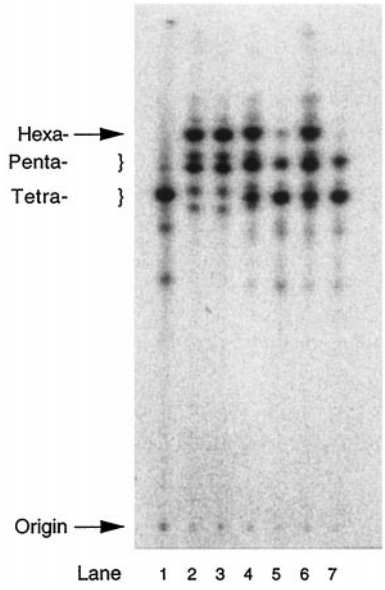

FIG. 4. **Tetra-acylated lipid A species that accumulate in *htrB* mutant MLK53 at 42 °C are not eliminated by extra copies of *msbA*.** Lipid A samples were isolated by hydrolysis in 0.2 M HCl from cultures labeled with $^{32}P_i$ for 3 h at either 30 or 42 °C, as described previously (19). Under these hydrolysis conditions both the Kdo and the 1-phosphate are released, giving rise to lipid A 4′-monophosphates (19, 29). This procedure also causes 10–15% *O*-deacylation. Approximately 1000 cpm of isolated lipid A ^{32}P-labeled 4′-monophosphates was applied to each lane of a Silica Gel 60 thin layer chromatography plate. The plate was developed in the solvent chloroform/pyridine/88% formic acid/water (55:50:16:5, v/v). The plate was then dried and exposed overnight to a PhosphorImager screen. The positions of the hexa-acylated (*Hexa-*), penta-acylated (*Penta-*), and tetra-acylated (*Tetra-*) lipid A ^{32}P-labeled 4′-monophosphates, as well as the origin, are indicated. *Lane 1* shows the position of a tetra-acylated lipid A ^{32}P-labeled 4′-monophosphate standard that was generated by acid hydrolysis of 4′-^{32}P-labeled lipid IV_A. *Even lanes* are lipid A ^{32}P-labeled 4′-monophosphates from cells grown at 30 °C and *odd lanes* from cells grown at 42 °C. The strains are as follows: *lanes 2* and *3,* W3110; *lanes 4* and *5*, *htrB* mutant MLK53; *lanes 6* and 7, MLK53/pKW2(*msbA*$^+$).

contained 25 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.1% Triton X-100, 5.5 μM [1-^{14}C]dipalmitoyl-phosphatidylcholine (~10,000 cpm/tube), and 2 μl of membrane sample in a final volume of 10 μl. After incubation at 37 °C for 60 min, the reaction mixture was converted to a two-phase Bligh and Dyer mixture by addition of 22 μl of chloroform/methanol (1:1, v/v). The lower phase was spotted onto a Silica Gel 60 high performance thin layer chromatography plate. The plate was developed in the solvent chloroform/methanol/water (65:25:4, v/v). The plate was dried and exposed overnight to a PhosphorImager screen. One unit of phospholipase A activity is defined as the amount of enzyme that generates 1 nmol of free fatty acid per min at 37 °C. NADH oxidase activity (an inner membrane marker) was measured spectrophotometrically (33). One unit of NADH oxidase activity is defined as 1 μmol of NADH oxidized per min at 25 °C.

Rapid Detection of Lipid A Molecular Species Released by pH 4.5 Hydrolysis of $^{32}P_i$-Labeled Membrane Fractions—A small amount of membrane fraction (usually several μl containing about 2,000 cpm) was diluted to 10 μl so that the final solution contained 12.5 mM sodium acetate, pH 4.5, and 1% SDS (34–36). This solution was incubated at 100 °C for 30 min in a 0.6-ml plastic microcentrifuge tube. The hydrolyzed material was converted to a two-phase Bligh and Dyer mixture by addition of 22 μl of chloroform/methanol (1:1, v/v). The entire lower phase was applied to a Silica Gel 60 TLC plate. The plate was developed in the solvent of chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v). The plate was dried and exposed overnight to a PhosphorImager screen to visualize both the glycerophospholipids (which are not degraded under these conditions) and lipid A (which is selectively released from the inner Kdo of the core without being dephosphorylated or deacylated).

Mass spectrometry of lipid A, purified after release from cells by pH 4.5 hydrolysis (36), was used to validate the identification of the radioactive lipids bands observed by thin layer chromatography. In wild type cells two forms of lipid A are recovered. Both are hexa-acylated, but a more hydrophilic form (about 30% of the total) contains an extra phosphate moiety, forming a diphosphate monoester (in place of the predominant monophosphate) at the 1-position of the proximal glucosamine residue (Fig. 2).

RESULTS

MsbA on pKW2 Suppresses the Temperature-sensitive Growth Phenotype of a Mutant Lacking htrB—Twelve colonies were isolated from a population of MLK53/pKW2 transformants selected on agar plates at 44 °C, and plasmid DNA was isolated from these strains. All of the colonies that grew at 44 °C contained pKW2, as determined by gel electrophoresis of the plasmid digested with *Eco*RV and *Hin*dIII. pACYC184 alone was unable to suppress the temperature-sensitive phenotype of MLK53.

As shown in Fig. 3, *htrB* mutant cells (MLK53) stop growing after about 4 h in shaking culture when shifted from 30 to 42 °C. Transformation of strain MLK53 with pKW2 also restored growth at 42 °C on liquid LB medium (albeit somewhat slower than wild type). These findings show that the *orfE/lpxK* gene downstream of *msbA* (21) is not needed for the suppression of the *htrB* mutant phenotype.

MsbA Does Not Increase the Extent of Lipid A Acylation in htrB- deficient Mutants at 42 °C—Lipid A of wild type cells is acylated mostly with six fatty acids irrespective of growth temperature, as judged by $^{32}P_i$ labeling and recovery of the lipid A 4′-monophosphates following strong acid hydrolysis (Fig. 4, *lanes 2* and *3*) (19). The *msbB* mutation (not shown in Fig. 4) blocks the last acylation of lipid A biosynthesis, resulting in the accumulation of the penta-acylated species (19, 23). Five acyl chains on lipid A are sufficient to support growth (19, 23). However, the *htrB* mutant MLK53 grown at 42 °C generates mainly tetra-acylated lipid A moieties (Fig. 4, *lane 5*). When grown at 30 °C, *htrB* mutants do synthesize some penta- and hexa-acylated lipid A moieties (19) (Fig. 4, *lane 4*), presumably by employing alternative acylation pathways, such as palmitoleate incorporation by Ddg (37) or palmitate transfer from glycerophospholipids (38). These additional acylation pathways do not seem to operate efficiently at 42 °C. As shown in Fig. 4 (*lanes 6* and 7), introduction of pKW2 into *htrB* deficient cells does not increase the extent of acylation of lipid A either at 30 or 42 °C, even though the mutant cells are now able to grow at 42 °C (Fig. 3). These findings demonstrate that

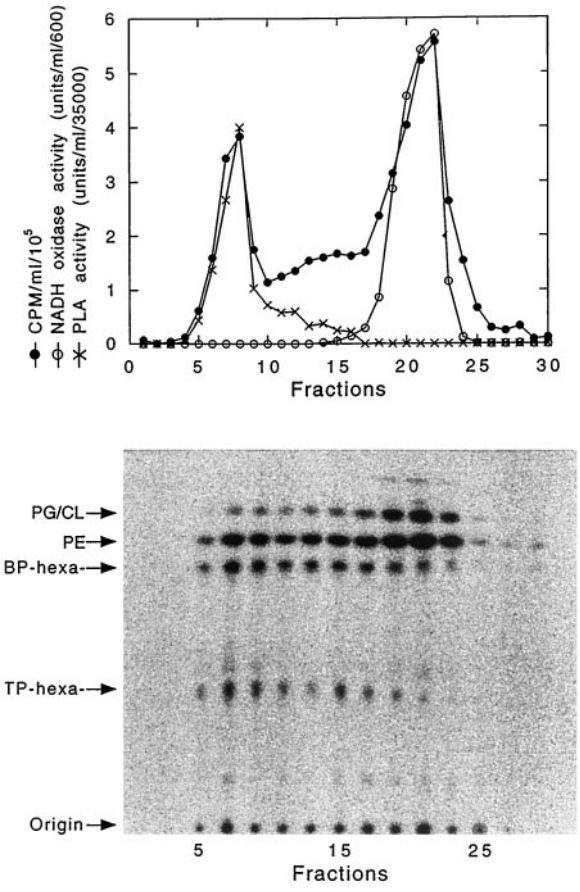

FIG. 5. **Rapid detection of hexa-acylated lipid A species and glycerophospholipids in inner and outer membranes of wild type *E. coli* W3110 grown at 42 °C.** Overnight cultures grown at 30 °C were diluted with fresh pre-warmed LB broth to A_{550} of 0.05. After addition of 4 μCi/ml $^{32}P_i$, cells were grown at 42 °C until A_{550} = 0.6. Membranes were prepared and analyzed as described under "Experimental Procedures." Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/600), and *crosses* (units/ml/35,000), respectively. Lipid A was released by pH 4.5 hydrolysis and quantified together with glycerophospholipids by thin layer chromatography and PhosphorImager analysis, as described under "Experimental Procedures" (*lower panel*). The positions of phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*), the hexa-acylated lipid A bis- and trisphosphates (*BP-hexa-* and *TP-hexa-*, respectively), as well as the origin, are indicated (*lower panel*). *Upper* and *lower panels* have been arranged in such a way that each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*. Every other fraction was processed and spotted.

MsbA is not an alternative acyltransferase that can compensate for the absence of HtrB.

Rapid Detection of Lipid A Molecular Species in Separated Inner and Outer Membranes of Wild Type and Mutant Strains—In order to compare the composition and subcellular localization of lipid A species in various mutant strains, inner and outer membranes were separated by isopycnic sucrose gradient centrifugation (30) from cells that had been grown in the presence of $^{32}P_i$, as described under "Experimental Procedures." Inner membranes were detected by assaying NADH oxidase, and outer membranes were located by determining the activity of phospholipase A in each fraction, as shown in Fig. 5 (*upper panel*) for the wild type strain W3110 grown at 42 °C. Excellent separation was observed. The total amount of ^{32}P in each fraction was determined by liquid scintillation counting (Fig. 5, *upper panel*). Lipid A species were then released from the labeled membrane fragments by pH 4.5 hydrolysis (a milder procedure than the one used in the experiment of Fig. 4) that breaks the Kdo-lipid A linkage (Fig. 2) without causing 1-dephosphorylation or deacylation (34–36). Membrane glycerophospholipids also are retained during pH 4.5 hydrolysis. As shown in Fig. 5 (*lower panel*), chloroform extraction of the pH 4.5 hydrolyzed membranes, followed by thin layer chromatography and PhosphorImager analysis, allowed simultaneous detection of the major glycerophospholipids (phosphatidylethanolamine and phosphatidylglycerol plus cardiolipin) and the two major hexa-acylated lipid A molecular species present in wild type cells. These two forms of hexa-acylated lipid A differ only by the presence of an additional phosphate residue. The more slowly migrating form corresponds to the trisphosphate species. The molecular weights of these lipid A species were confirmed by mass spectrometry (36).[2] Both the lipid A bisphosphate and the trisphosphate species (Fig. 5, *BP* and *TP*, respectively) were localized mainly in the outer membrane. The structures of the hexa-acylated lipid A bisphosphate and trisphosphate species are shown at the *bottom* of Fig. 2.

The glycerophospholipids (39) were identified by their migration with commercial standards. A higher percentage of phosphatidylethanolamine compared with phosphatidylglycerol plus cardiolipin was observed in the outer membrane compared with the inner membrane (Fig. 5), consistent with reported compositions (40). Similar results to those in Fig. 5 were obtained with other wild type strains irrespective of growth temperature (data not shown).

Localization of Penta-acylated Lipid A Species in the Outer Membranes of msbB Mutants Grown at 42 °C—Mutant MLK1067 (*msbB*$^-$), which is not temperature-sensitive, is defective in the enzyme that incorporates the sixth fatty acid present on lipid A, usually a myristate residue (Fig. 2) (19, 20). MLK1067 was labeled at 42 °C for several generations. The membrane separation and the thin layer analysis of the hydrolyzed fractions are shown in Fig. 6. In MLK1067, penta-acylated lipid A species predominate as both the bis- and the trisphosphates (Fig. 6). Both forms of lipid A in MLK1067 are located mainly in the outer membranes, as judged by the phospholipase A profile (Fig. 6). Mass spectrometry of the lipid A isolated from *msbB*-deficient cells (23) reveals the absence of the myristate moiety normally present on the distal subunit of lipid A (Fig. 2).

Accumulation of Tetra-acylated Lipid A Species in the Inner Membranes of htrB Mutants Shifted to 42 °C—MLK53 (*htrB*$^-$) cannot grow above 32.5 °C (16, 17). MLK53 was labeled with $^{32}P_i$ either at 30 or at 42 °C for 3 h. The membrane separations and the lipid profiles of the individual fractions are shown for cells grown at 30 °C (Fig. 7) or shifted to 42 °C (Fig. 8). In 30 °C grown cells, the membranes were well resolved (Fig. 7). Most of the lipid A was present in the outer membranes, either as the bis- or trisphosphates of penta- and hexa-acylated species. As noted above, other enzymes, such as Ddg (37) or the one that transfers palmitate from glycerophospholipids to lipid A (38), may substitute for the absence of HtrB at 30 °C, allowing for the generation of more highly acylated lipid A molecules. At 42 °C, however, almost all the newly synthesized lipid A (and most of the glycerophospholipids) accumulate in the inner membrane (Fig. 8). The membranes are not as well resolved as those isolated from 30 °C grown cells (Fig. 7), but they are nevertheless discernible (Fig. 8). There also appears to be a relative increase in the amount of glycerophospholipids (Fig. 8), consistent with the previous observation that the phospholipid to protein ratio doubles in *htrB* mutants at non-permissive temperatures (18). Very little newly synthesized lipid of any

[2] Z. Zhou, I. Kaltashov, and C. R. H. Raetz, unpublished observations.

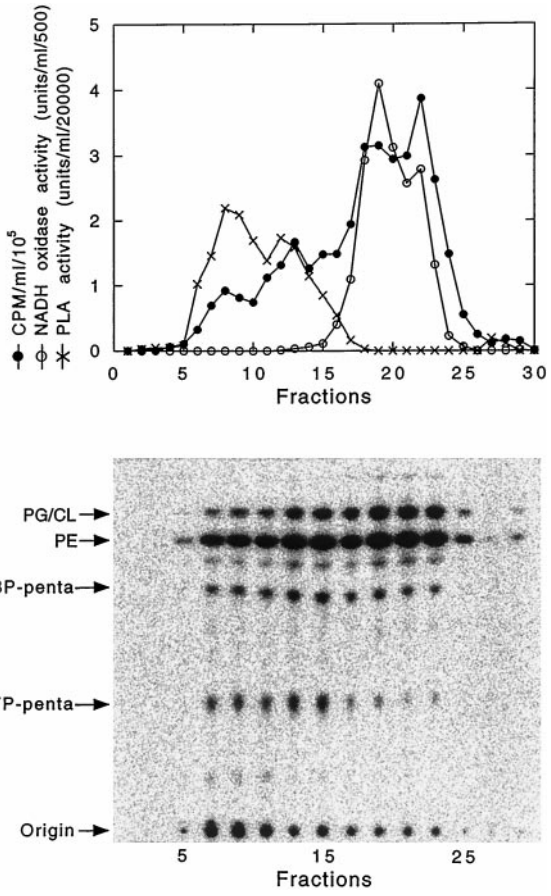

FIG. 6. **Penta-acylated lipid A species and glycerophospholipids in inner and outer membranes of *E. coli* mutant MLK1067(*msbB*$^-$) grown at 42 °C.** As this strain is not temperature-sensitive for growth, it was labeled and analyzed exactly as described in Fig. 5. Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/500), and *crosses* (units/ml/20,000), respectively. The positions of phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*), the penta-acylated lipid A bis- and trisphosphates (*BP-penta-* and *TP-penta-*, respectively), as well as the origin, are indicated (*lower panel*). Each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*, as in Fig. 5. The steady-state level of lipid A species in the inner membranes of *msbB* mutants may be slightly higher than in wild type cells (Fig. 5).

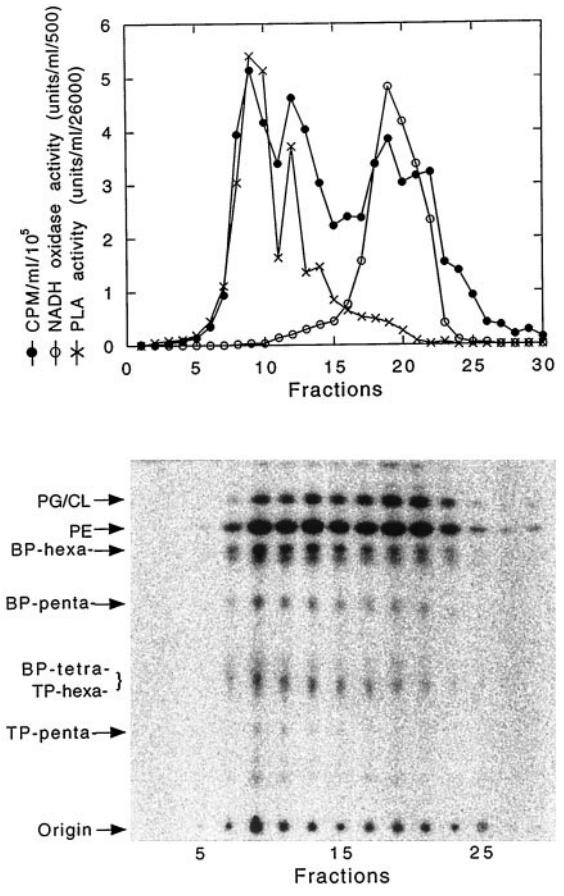

FIG. 7. **Lipid A molecular species and glycerophospholipids in inner and outer membranes of *E. coli* mutant MLK53(*htrB*$^-$) grown at 30 °C.** This strain was labeled for 3 h at 30 °C starting at A_{550} of 0.05, and membranes were prepared and analyzed as described in Fig. 5. Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/500), and *crosses* (units/ml/26,000), respectively. The positions of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), the hexa- and penta-acylated lipid A bis- and trisphosphates, as well as the origin, are indicated (*lower panel*). Each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*, as in Fig. 5.

kind reaches pre-existing outer membranes at 42 °C (as judged by the phospholipase A profile in Fig. 8). Predominantly tetra-acylated lipid A bisphosphate (but very little trisphosphate) was found in association with the inner membranes, which are more heterogeneous in buoyant density than wild type. None of the accumulated tetra-acylated lipid A species could be extracted directly from the inner membranes of *htrB* mutants using chloroform/methanol mixtures without prior pH 4.5 hydrolysis (data not shown). These findings indicate that these abnormal lipid A species accumulating at 42 °C probably contain a substantial core oligosaccharide, given that tetra-acylated precursors lacking a complete core, such as Kdo_2-lipid IV_A, are chloroform/methanol-soluble without prior pH 4.5 hydrolysis (41). Accumulation of underacylated lipid A-core molecules in the inner membrane may be toxic and account for the inability of *htrB* mutant bacteria to grow at 42 °C.

Effects of Extra Copies of msbA on the Membrane Composition of htrB Mutants—The *msbA* gene was discovered as a multi-copy suppressor of *htrB* mutants (21). In order to understand how *msbA* rescues *htrB* mutants at 42 °C, the *htrB* mutant MLK53 was transformed with the *msbA* bearing plasmid (pKW2) to generate strain MLK53/pKW2. MLK53/pKW2 cells were then grown and labeled for 3 h with $^{32}P_i$ at 42 °C. The membrane separations and the radioactive lipid profiles are shown in Fig. 9. The lipid A species present in the MLK53/pKW2 membranes are similar to those found in MLK53 grown at 42 °C (Fig. 8), but a significant fraction of the tetra-acylated lipid A species are recovered with the outer membranes. The formation of a tetra-acylated trisphosphate is also observed in MLK53/pKW2 in conjunction with transport to the outer membrane. A small amount of residual penta-acylated lipid A is also observed, as in Fig. 4. These observations support the view that MsbA is a lipid A transporter rather than functioning as a distinct lipid A acyltransferase.

Accumulation of Lipid A Species in the Inner Membranes of htrB$^+$ *Cells When msbA and orfE Are Not Expressed*—Strain AP191 contains an insertion in the chromosomal *msbA* gene, which also inactivates the downstream *orfE*/*lpxK* gene (21). In addition, *msbA*$^+$ and *lpxK*$^+$ are present on a plasmid with a temperature-sensitive origin of replication (24). After several hours at 42 °C, LPS accumulates in the inner membranes of such constructs, as judged by *N*-acetyl[^{3}H]glucosamine labeling (24). To confirm these findings with our ^{32}P labeling technique, membrane separations and lipid A analyses were performed on both AP191 (as described in Fig. 10) and on a matched strain,

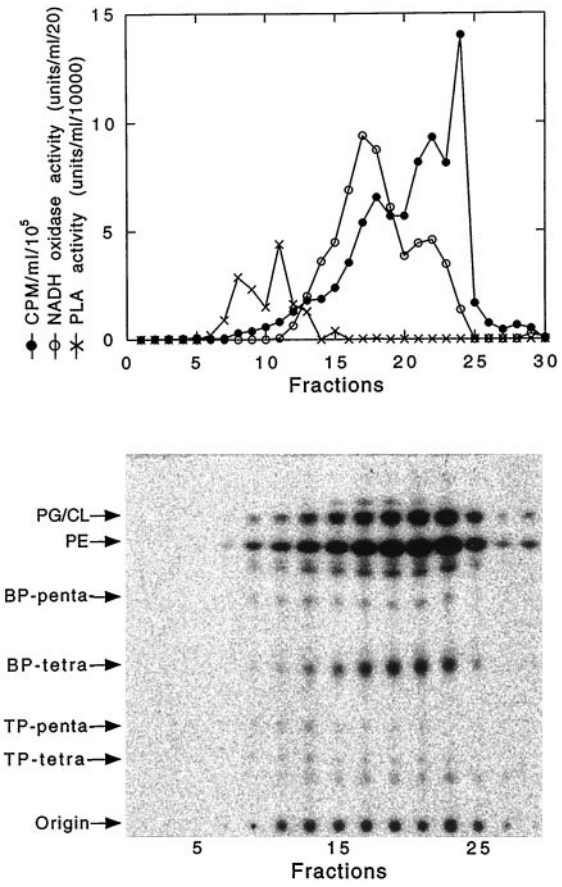

FIG. 8. **Accumulation of tetra-acylated lipid A species in the inner membranes of *E. coli* mutant MLK53(*htrB*$^-$) shifted to 42 °C.** Overnight cultures grown at 30 °C were diluted to A_{550} of 0.05 and then labeled for 3 h at 42 °C with $^{32}P_i$ at 4 μCi/ml. Membranes were prepared and analyzed as in Fig. 5. Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/20), and *crosses* (units/ml/10,000), respectively. The positions of phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*), the penta- and tetra-acylated lipid A bis- and trisphosphates, as well as the origin, are indicated (*lower panel*). Each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*, as in Fig. 5.

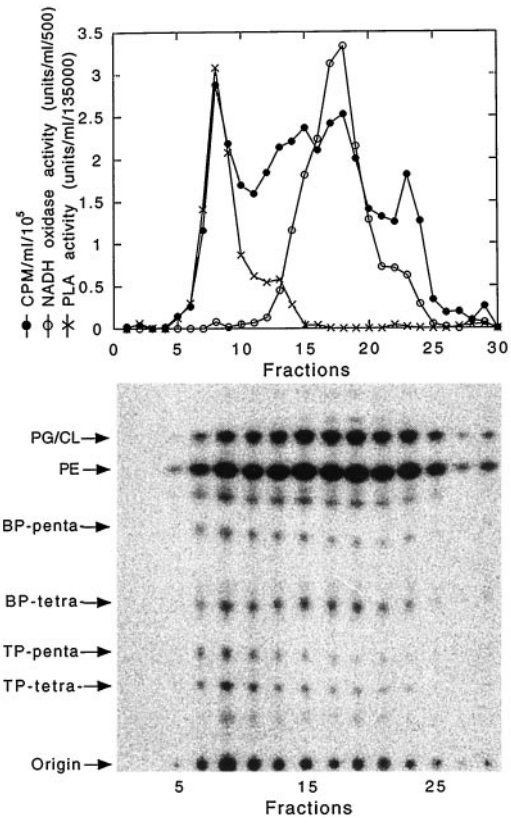

FIG. 9. **Translocation of tetra-acylated lipid A molecular species to outer membranes in MLK53(*htrB*$^-$)/pKW2(*msbA*$^+$) shifted to 42 °C.** Overnight cultures grown at 30 °C were diluted with fresh pre-warmed LB broth to A_{550} = 0.05. After addition of 4 μCi/ml $^{32}P_i$, cells were grown at 42 °C until A_{550} = 0.6. Membranes were isolated and analyzed as in Fig. 5. Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/500), and *crosses* (units/ml/135,000), respectively. The positions of phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*), the penta- and tetra-acylated lipid A bis- and trisphosphates, as well as the origin, are indicated (*lower panel*). Each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*, as in Fig. 5.

designated AP70, that bears a plasmid with a normal origin of replication (24) (data not shown). The membrane separation and the ^{32}P-lipid profiles of the fractions derived from cells of AP70 grown at 42 °C (data not shown) were very similar to those of W3110 (Fig. 5). AP191 was first shifted to 42 °C for 5 h to dilute out the *msbA*/*orfE* bearing plasmid, and then the cells were labeled with $^{32}P_i$ for 3 h (Fig. 10). Under these conditions almost all of the newly made radioactive lipids were located in the inner membranes (Fig. 10). Two lipid A-related compounds, not seen in the inner membranes of wild type cells (Fig. 5), also accumulated in the inner membranes of AP191 at 42 °C (Fig. 10). One of these could not be extracted with chloroform/methanol mixtures without prior pH 4.5 hydrolysis and was identified as hexa-acylated lipid A bisphosphate (Fig. 10). The other lipid that accumulated was slightly more hydrophobic but could be extracted directly with chloroform/methanol mixtures. As shown in Fig. 10, this material had the same apparent mobility as a disaccharide 1-phosphate (DS-1-P) standard, the intermediate of the lipid A pathway that is the substrate for the 4′-kinase (Fig. 2) (26, 42, 43). Since *orfE*/*lpxK* has recently been shown to encode the 4′-kinase (26), the accumulation of DS-1-^{32}P in the inner membranes of AP191 at 42 °C can be explained by the loss of *orfE*/*lpxK*.

Absence of MsbA Alone Causes Lipid A Accumulation in Inner Membranes—To exclude the possibility that the inner membrane build up of hexa-acylated lipid A bisphosphate observed in AP191 at 42 °C (Fig. 10) might represent an indirect effect of DS-1-P accumulation on lipid A transport, instead of being due to the loss of MsbA *per se*, AP191 was transformed with the *orfE* bearing hybrid plasmid, pEH2. The latter is compatible with the temperature-sensitive hybrid plasmid (bearing *msbA* and *orfE*) present in AP191. Furthermore, pEH2 is maintained and can express the 4′-kinase irrespective of the temperature at which the cells are grown. AP191/pEH2 displays the same temperature-sensitive growth characteristics as does AP191 (data not shown). Cells of AP191/pEH2 were therefore labeled in the same way as described for AP191 in Fig. 10, and the membranes were separated and analyzed for lipid A molecular species (Fig. 11). As observed with AP191, most of the lipid radioactivity of AP191/pEH2 present after 3 h labeling at 42 °C was located in the inner membranes, but now only the hexa-acylated lipid A bisphosphate accumulated (Fig. 11). DS-1-P was not detectable. The results indicate that the absence of MsbA alone can account for the accumulation of hexa-acylated lipid A species within the inner membrane and that the effects of *orfE* inactivation in AP191 (Fig. 10) are peripheral. As in *htrB* mutants shifted to 42 °C, no lipid A trisphosphates are observed when hexa-acylated lipid A fails to

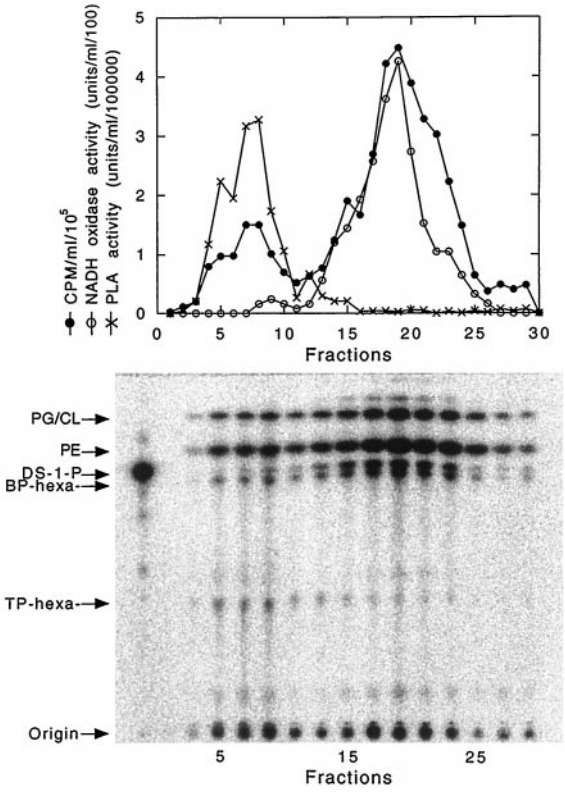

FIG. 10. **Accumulation of hexa-acylated lipid A molecular species, DS-1-P, and glycerophospholipids in inner membranes of *E. coli* AP191, lacking both MsbA and OrfE at 42 °C.** Overnight cultures grown at 30 °C were diluted with fresh pre-warmed LB broth to A_{550} = 0.05. AP191 cells were allowed to grow at 42 °C for 5 h to dilute out MsbA and OrfE/LpxK. Cells were diluted 10-fold into fresh LB medium whenever A_{550} reached 0.6. Then, cells in 125 ml of LB broth with A_{550} ~0.3 were labeled with 4 μCi/ml $^{32}P_i$ at 42 °C for 3 more h. Membrane separations and lipid analyses were performed as in Fig. 5. Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/100), and *crosses* (units/ml/100,000), respectively. The positions of phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*), DS-1-P (the 4′-kinase substrate shown in Fig. 2), the hexa-acylated lipid A bis- and trisphosphates, as well as the origin, are indicated (*lower panel*). Each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*, as in Fig. 5.

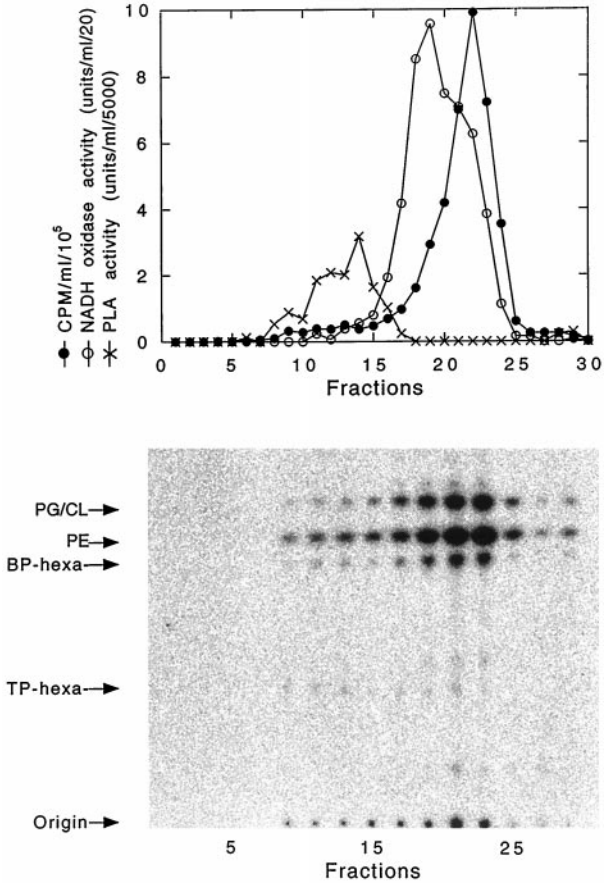

FIG. 11. **Accumulation of hexa-acylated lipid A molecular species and glycerophospholipids in inner membranes of *E. coli* AP191/pEH2, lacking only MsbA at 42 °C.** Cells were grown, labeled, and analyzed as in Fig. 10. Total radioactivity, NADH oxidase activity, and phospholipase A activity in each fraction (*upper panel*) were measured and are represented as *filled circles* (cpm/ml/10^5), *open circles* (units/ml/20), and *crosses* (units/ml/5000), respectively. The positions of phosphatidylethanolamine (*PE*), phosphatidylglycerol (*PG*), cardiolipin (*CL*), and the hexa-acylated lipid A bis- and trisphosphates, as well as the origin, are indicated (*lower panel*). Each lane in the *lower panel* is aligned with its corresponding fraction in the *upper panel*, as in Fig. 5.

reach the outer membrane in AP191/pEH2 (Fig. 11). Taken together, these findings lend additional support to the idea that MsbA may function as a transporter of newly synthesized lipid A-core molecules in *E. coli* (24, 25).

DISCUSSION

The *msbA* gene was discovered because multiple copies of *msbA* suppress the temperature-sensitive growth of *htrB* mutants (Fig. 2) (21). The connection to the LPS system was made in 1995 with the recognition that *htrB* is the structural gene for the lauroyltransferase of the lipid A pathway (Fig. 2) (15, 16). The fact that MsbA is highly homologous to a large family of ABC transport proteins and is required for the growth of *E. coli* raised the intriguing possibility that MsbA might play a role in LPS secretion, a process about which little is known (Fig. 1) (21).

In the present work, we have made the following new observations. (*a*) Tetra-acylated lipid A species accumulate in the inner membranes of *htrB* mutants at 42 °C, but *htrB* mutants grown at 30 °C are able to make penta- and hexa-acylated lipid A molecules, presumably using alternative acyltransferases. (*b*) *msbA* does not increase the extent of lipid A acylation in *htrB* mutants. (*c*) Extra copies of *msbA* introduced into *htrB* mutants stimulate the transport of a large fraction of the tetra-acylated lipid A species to the outer membrane at 42 °C. (*d*) Hexa-acylated lipid A species accumulate in the inner membranes of cells depleted of MsbA, even when LpxK depletion is prevented by the presence of an additional *lpxK* bearing plasmid. (*e*) Glycerophospholipids accumulate together with lipid A in the inner membranes of both *htrB* and *msbA* mutant cells at 42 °C, suggesting that MsbA might also play a role in glycerophospholipid transport. (*f*) When lipid A fails to reach the outer membrane, the trisphosphate form of lipid A is not observed.

LPS had recently been shown to accumulate in the inner membranes of mutants with disrupted *htrB* and *msbA* genes, as judged by gel electrophoresis, pulse labeling with *N*-acetyl[^{3}H]glucosamine (24), or antibody binding (25). However, no unambiguous way to quantify the amount of lipid A and to determine the extent of its acylation in isolated membrane vesicles had been described. To address this problem, we devised a rapid, new procedure for detecting ^{32}P-labeled lipid A residues in small biological samples. Our assay is based on the observation that the Kdo-lipid A linkage (Fig. 2) is cleaved selectively by pH 4.5 hydrolysis at 100 °C in the presence of SDS without measurable loss of ester-linked fatty acids or of the anomeric phosphate (34–36). The hydrolysis procedure can be applied directly to radioactive cells or to isolated membrane fractions from a sucrose gradient without first extracting LPS with

phenol-containing solvents. The lipid A released in this manner is recovered by Bligh-Dyer extraction together with the glycerophospholipids, which also remain intact throughout the procedure. The lipid A and the glycerophospholipids can then be separated and quantified simultaneously by TLC analysis (Fig. 5, *lower panel*). The identification of the hexa-acylated lipid A bis- and trisphosphate spots (Fig. 5, *BP-hexa* and *TP-hexa*, respectively) was validated by mass spectrometry (36), in conjunction with ^{1}H, ^{31}P, and ^{13}C NMR spectroscopy.[3] In several wild type *E. coli* strains grown at 30 or 42 °C (not shown), the lipid A is largely hexa-acylated and is mainly recovered in the outer membrane fractions (Fig. 5). The small amount of lipid A found in the inner membranes (Fig. 5) may represent newly synthesized core-lipid A, given that the cells were labeled with $^{32}P_i$ for several generations.

Previous genetic studies showed that both *msbA* and *orfE/lpxK* are essential *E. coli* genes (21). Consistent with previous observations (21), the experiment of Fig. 3 shows that the phenotypic suppression of the *htrB* mutation can be attributed solely to *msbA*. Interestingly, the growth rate of the suppressed mutant is slower than wild type. Slow growth may be explained by the finding that *msbA* does not correct the lipid A acylation deficiency associated with the *htrB* mutation (Fig. 4). The tetra-acylated lipid A precursors that accumulate at 42 °C in *htrB* mutants may not be rapidly transported, given their accumulation in the inner membrane (Fig. 8). The tetra-acylated lipid A species that accumulate in the inner membranes of *htrB* mutants at 42 °C probably contain a complete core, as pH 4.5 hydrolysis is required to recover these species by Bligh-Dyer extraction (data not shown). Overexpression of *msbA* results in the translocation of a significant fraction of the tetra-acylated lipid A species to the outer membrane (Fig. 9). These findings support the view that MsbA acts by stimulating lipid A transport and that it is not likely to be an acyltransferase.

The *htrB* mutant MLK53 grown at 30 °C synthesizes large amounts of penta- and hexa-acylated lipid A moieties (19) (Figs. 4 and 7), which are efficiently translocated to the outer membrane. The origin of these acylated species is not entirely clear, given that the *htrB* gene in MLK53 is disrupted by an insertion (17). Some of this acylation may be catalyzed by Ddg (a cold-shock induced palmitoleoyltransferase with homology to HtrB that appears to acylate the same OH group on Kdo_2-lipid IV_A normally employed by HtrB) (37). Ddg-catalyzed acylation could be followed by the action of MsbB (Fig. 2) to generate a hexa-acylated lipid A moiety. Alternatively, the enzyme that transfers palmitate residues from glycerophospholipids to lipid A precursors (38) (the structural gene of which is unknown) may be activated in *htrB* mutants. Mass spectrometry of lipid A isolated from MLK53 grown at 30 °C (data not shown) suggests that palmitoleate and myristate attachment account for most of the observed penta- and hexa-acylated lipid A species (Figs. 4 and 7). Mass spectrometry of lipid A isolated from *Salmonella typhimurium* strains lacking *htrB* likewise suggests the activation of compensatory lipid A acylation reactions when such mutants are grown at 30 °C (44). Mutants lacking both *htrB* and these other acyltransferases may therefore be unable to grow at any temperature in the absence of a covering plasmid carrying *msbA*.

Five acyl chains on lipid A seem to be sufficient to support the growth of *E. coli* cells containing only a single chromosomal copy of *msbA* (19, 23). The growth rate of strains defective in *msbB* (Fig. 2) is nearly normal at 30 and 42 °C, despite the fact that their lipid A consists almost entirely of penta-acylated species. The latter, like the penta-acylated lipid A moieties seen in *htrB* mutants grown at 30 °C (Fig. 7), are translocated efficiently to the outer membrane (Fig. 6). Taken together, these findings indicate that one acyloxyacyl group on lipid A may be needed for proper recognition by the secretion machinery.

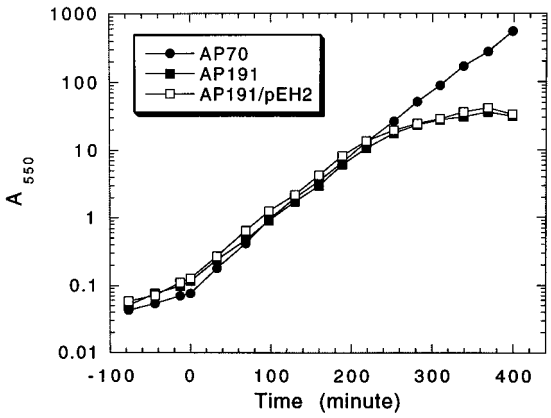

FIG. 12. **Temperature-sensitive growth of strains depleted of *msbA*.** Strains were grown as described in Fig. 3, except that the antibiotic spectinomycin was left out of the LB broth when the temperature was shifted to 42 °C. It took about 5 h to dilute out the covering plasmid that contains both *msbA* and *orfE/lpxK* (24).

Hexa-acylated lipid A species accumulate in the inner membranes of strains depleted of MsbA, which cannot grow because MsbA is essential (Figs. 10 and 11). In these experiments, the depletion of MsbA is achieved by shifting a strain containing a chromosomal insertion mutation in *msbA* and a temperature-sensitive covering plasmid bearing $msbA^{+}$ to 42 °C. MsbA depletion takes 3–5 h in the available constructs. Growth inhibition is very gradual (Fig. 12). The cells nevertheless take up reasonable amounts of $^{32}P_i$ from the medium during the 3-h labeling period after MsbA is diluted out, and they incorporate it into all major membrane lipids (as judged by the total counts recovered in Figs. 10 or 11 *versus* Fig. 5). Efficient labeling under these conditions argues that the effects of MsbA depletion are not indirect. The lack of the *msbA* covering plasmid during the period of $^{32}P_i$ labeling in the experiments of Figs. 10 and 11 was confirmed by plating the cells in the presence of 50 μg/ml spectinomycin and showing that the number of colonies was reduced by 4 orders of magnitude compared with what was expected from the optical density of the culture.

A problem with the published MsbA depletion experiments, as well as the experiment in Fig. 10, is that *msbA* is an upstream gene in an operon (24, 25). The essential downstream gene (*orfE/lpxK*), now known to encode the 4′-kinase of lipid A biosynthesis (Fig. 2) (26), is inactivated together with *msbA*. Expression of extra *lpxK* genes on a separate plasmid in MsbA-depleted cells does not prevent lipid A accumulation in the inner membranes or gradual growth arrest (Figs. 11 and 12). Thus, lipid A accumulation in the inner membrane can be attributed solely to MsbA depletion (Fig. 11). The only difference is that the 4′-kinase substrate (DS-1-P) no longer accumulates when LpxK depletion is prevented (Fig. 11 *versus* Fig. 10). The identification of DS-1-P in Fig. 10 was confirmed by the finding that pH 4.5 hydrolysis was not required for efficient Bligh-Dyer extraction (data not shown). Recently, we have demonstrated that selective *lpxK* inactivation also results in growth arrest and DS-1-P accumulation (53). The effects of selective LpxK depletion on lipid A transport have not yet been examined. Therefore, we cannot exclude the possibility that MsbA and LpxK are both components of the putative core-lipid A transporter (Fig. 1).

The biosynthetic origin of the trisphosphate species of lipid A (45) (Figs. 5, 6, 7 and 9) has not been established. The trisphos-

[3] Z. Zhou and C. R. H. Raetz, manuscript in preparation.

phate is not observed when lipid A transport is blocked (Figs. 8, 10, and 11). This interesting finding suggests that the trisphosphate is generated on the periplasmic surface of the inner membrane or in the outer membrane. Since ATP would not be available in the outer parts of the envelope, other donors of high energy phosphate moieties must be considered, such as bactoprenol-pyrophosphate, a product of O-antigen polymerization (Fig. 1) (4), or diacylglycerol-pyrophosphate, a newly discovered, minor lipid (46). Attempts to generate trisphosphate *in vitro* using ATP and Kdo_2-lipid IV_A have been unsuccessful.[4]

Our assays (Figs. 8, 10, and 11) indicate that glycerophospholipids accumulate together with lipid A in the inner membrane when lipid A transport is blocked in *htrB*- or *msbA*-deficient strains. One provocative explanation is that MsbA transports both lipid A and glycerophospholipids. This idea is consistent with several of the following observations. (*a*) The *msbA* gene (21) is a member of a large subset of ABC transporters of largely unknown function found in all eubacteria, archaebacteria, and eucaryotic cells. Multiple copies of *msbA*-like genes (coding for proteins of ~590 amino acids) are present in all genomes, as judged by BLAST searching (47, 48). Since lipid A is found only in Gram-negative bacteria (2, 3), the wide distribution of *msbA* genes in all organisms suggests a more general function for MsbA. (*b*) The high sequence homology of *E. coli msbA* to the two homologous domains of mammalian Mdr proteins is especially striking (about 10^{-84} and 10^{-72} using the Gapped-Blast algorithm) (48). This score is comparable to the similarity of *E. coli msbA* to *msbA* genes found in other Gram-negative bacteria (48). (*c*) The function of Mdr-2 in the mouse has recently been determined by construction of a strain in which the *mdr-2* gene is knocked out (49, 50). This mouse displays a 10-fold reduction in the secretion of phosphatidylcholine into its bile, consistent with the high level of expression of Mdr-2 in the cells lining the biliary tree (49, 50). This finding shows that Mdr proteins are capable of transporting specific glycerophospholipids in a physiologically relevant setting (49, 50). The multiplicity of *msbA*-like genes in nature may therefore reflect the requirement for protein-mediated transport (*i.e.* flip-flop) of diverse lipid molecules across biological membranes.

To prove unequivocally that MsbA functions as a general membrane lipid transporter in *E. coli*, additional mutants defective in *msbA* need to be isolated, and more informative biochemical assays must be devised. For instance, a mutant allele of *msbA* that results in the complete loss of MsbA activity within minutes after a shift to 42 °C would be very helpful, since such a strain should accumulate newly made lipid A and glycerophospholipids within its inner membranes shortly after a temperature shift. The time course of inactivation of other secretory events and of macromolecular synthesis could then be assessed and should help establish what effects are primary and secondary. Lastly, MsbA needs to be purified to homogeneity and reconstituted into model membrane vesicles in order to demonstrate conclusively its ability to catalyze ATP-dependent lipid A and glycerophospholipid flip-flop.

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REFERENCES

1. Nikaido, H. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) Vol. 1, 2nd Ed., pp. 29–47, American Society for Microbiology, Washington, D. C.
2. Raetz, C. R. H. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) Vol. 1, 2nd Ed., pp. 1035–1063, American Society for Microbiology, Washington, D. C.
3. Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zähringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) *FASEB J.* **8,** 217–225
4. Schnaitman, C. A., and Klena, J. D. (1993) *Microbiol. Rev.* **57,** 655–682
5. Morrison, D. C., and Ryan, J. L. (eds) (1992) *Bacterial Endotoxic Lipopolysaccharides*, Vol. 1, CRC Press, Inc., Boca Raton, FL
6. Raetz, C. R. H. (1993) *J. Bacteriol.* **175,** 5745–5753
7. Raetz, C. R. H. (1990) *Annu. Rev. Biochem.* **59,** 129–170
8. Reeves, P. (1994) *New Compr. Biochem.* **27,** 281–314
9. Mulford, C. A., and Osborn, M. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1159–1163
10. Osborn, M. J. (1979) in *Bacterial Outer Membranes* (Inouye, M., ed) pp. 15–34, John Wiley & Sons, Inc., New York
11. Marino, P. A., Phan, K. A., and Osborn, M. J. (1985) *J. Biol. Chem.* **260,** 14965–14970
12. Marino, P. M., McGrath, B. C., and Osborn, M. J. (1991) *J. Bacteriol.* **173,** 3128–3133
13. McGrath, B. C., and Osborn, M. J. (1991) *J. Bacteriol.* **173,** 649–654
14. McGrath, B. C., and Osborn, M. J. (1991) *J. Bacteriol.* **173,** 3134–3137
15. Clementz, T., Bednarski, J., and Raetz, C. R. H. (1995) *FASEB J.* **9,** 1311
16. Clementz, T., Bednarski, J. J., and Raetz, C. R. H. (1996) *J. Biol. Chem.* **271,** 12095–12102
17. Karow, M., Fayet, O., Cegielska, A., Ziegelhoffer, T., and Georgopoulos, C. (1991) *J. Bacteriol.* **173,** 741–750
18. Karow, M., Fayet, O., and Georgopoulos, C. (1992) *J. Bacteriol.* **174,** 7407–7418
19. Clementz, T., Zhou, Z., and Raetz, C. R. H. (1997) *J. Biol. Chem.* **272,** 10353–10360
20. Karow, M., and Georgopoulos, C. (1992) *J. Bacteriol.* **174,** 702–710
21. Karow, M., and Georgopoulos, C. (1993) *Mol. Microbiol.* **7,** 69–79
22. Karow, M., and Georgopoulos, C. (1991) *Mol. Microbiol.* **5,** 2285–2292
23. Somerville, J. E., Jr., Cassiano, L., Bainbridge, B., Cunningham, M. D., and Darveau, R. P. (1996) *J. Clin. Invest.* **97,** 359–365
24. Polissi, A., and Georgopoulos, C. (1996) *Mol. Microbiol.* **20,** 1221–1233
25. McDonald, M. K., Cowley, S. C., and Nano, F. E. (1997) *J. Bacteriol.* **179,** 7638–7643
26. Garrett, T. A., Kadrmas, J. L., and Raetz, C. R. H. (1997) *J. Biol. Chem.* **272,** 21855–21864
27. Miller, J. R. (1972) *Experiments in Molecular Genetics*, p. 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc., New York
29. Galloway, S. M., and Raetz, C. R. H. (1990) *J. Biol. Chem.* **265,** 6394–6402
30. Guy-Caffey, J. K., Rapoza, M. P., Jolley, K. A., and Webster, R. E. (1992) *J. Bacteriol.* **174,** 2460–2465
31. Osborn, M. J. (1974) *Methods Enzymol.* **31,** 642–653
32. Nishijima, M., Nakaike, S., Tamori, Y., and Nojima, S. (1977) *Eur. J. Biochem.* **73,** 115–124
33. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247,** 3962–3972
34. Caroff, M., Tacken, A., and Szabó, L. (1988) *Carbohydr. Res.* **175,** 273–282
35. Karibian, D., Deprun, C., and Caroff, M. (1993) *J. Bacteriol.* **175,** 2988–2993
36. Odegaard, T. J., Kaltashov, I. A., Cotter, R. J., Steeghs, L., van der Ley, P., Khan, S., Maskell, D. J., and Raetz, C. R. H. (1997) *J. Biol. Chem.* **272,** 19688–19696
37. Carty, S. M., Sreekumar, K., and Raetz, C. R. H. (1997) *FASEB J.* **11,** 1423
38. Brozek, K. A., Bulawa, C. E., and Raetz, C. R. H. (1987) *J. Biol. Chem.* **262,** 5170–5179
39. Raetz, C. R. H., and Dowhan, W. (1990) *J. Biol. Chem.* **265,** 1235–1238
40. Raetz, C. R. H., and Newman, K. F. (1979) *J. Bacteriol.* **137,** 860–868
41. Brozek, K. A., Hosaka, K., Robertson, A. D., and Raetz, C. R. H. (1989) *J. Biol. Chem.* **264,** 6956–6966
42. Ray, B. L., Painter, G., and Raetz, C. R. H. (1984) *J. Biol. Chem.* **259,** 4852–4859
43. Ray, B. L., and Raetz, C. R. H. (1987) *J. Biol. Chem.* **262,** 1122–1128
44. Sunshine, M., Gibson, B. W., Engstrom, J. J., Nichols, W. A., Jones, B. D., and Apicella, M. A. (1997) *J. Bacteriol.* **179,** 5521–5533
45. Strain, S. M., Fesik, S. W., and Armitage, I. M. (1983) *J. Biol. Chem.* **258,** 13466–13477
46. Dillon, D. A., Wu, W.-I., Riedel, B., Wissing, J. B., Dowhan, W., and Carman, G. M. (1996) *J. Biol. Chem.* **271,** 30548–30553
47. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
48. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
49. Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) *Cell* **75,** 451–462
50. Oude Elferink, R. P. J., Ottenhoff, R., van Wijland, M., Smit, J. J. M., Schinkel, A. H., and Groen, A. K. (1995) *J. Clin. Invest.* **95,** 31–38
51. Liu, D., Cole, R. A., and Reeves, P. R. (1996) *J. Bacteriol.* **178,** 2102–2107
52. Clementz, T., and Raetz, C. R. H. (1991) *J. Biol. Chem.* **266,** 9687–9696
53. Garrett, T. A., Que, N. L. S., and Raetz, C. R. H. (1998) *J. Biol. Chem.* **273,** 12457–12465

[4] Z. Zhou, S. Basu, and C. R. H. Raetz, unpublished observations.

Function of *Escherichia coli* MsbA, an Essential ABC Family Transporter, in Lipid A and Phospholipid Biosynthesis

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