Function of the *Escherichia coli* msbB Gene, a Multicopy Suppressor of htrB Knockouts, in the Acylation of Lipid A

ACYLATION BY MsbB FOLLOWS LAURATE INCORPORATION BY HtrB*

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Overexpression of the *Escherichia coli* msbB gene on high copy plasmids suppresses the temperature-sensitive growth associated with mutations in the htrB gene. *htrB* encodes the lauroyl transferase of lipid A biosynthesis that acylates the intermediate (Kdo)₃-lipid Ι₄A (Brozek, K. A., and Raetz, C. R. H. (1990) *J. Biol. Chem.*** 265, 15410–15417). Since *msbB* displays 27.5% identity and 42.2% similarity to *htrB*, we explored the possibility that *msbB* encodes a related acyltransferase. In contrast to *htrB*, extracts of strains with insertion mutations in *msbB* are not defective in transferring laurate from lauroyl acyl carrier protein to (Kdo)₂-lipid Ι₄A. However, extracts of *msbB* mutants do not efficiently acylate the product formed by HtrB, designated (Kdo)₂-(lauroyl)-lipid Ι₄A. Extracts of strains harboring *msbB* bearing plasmids acylate (Kdo)₃-(lauroyl)-lipid Ι₄A very rapidly compared with wild type. We solubilized and partially purified MsbB from an overproducing strain, lacking HtrB. MsbB transfers myristate or laurate, activated on ACP, to (Kdo)₂-(lauroyl)-lipid Ι₄A. Decanoyl, palmitoyl, palmitoleoyl, and (R)-3-hydroxymyristoyl-ACP are poor acyl donors. MsbB acylates (Kdo)₂-(lauroyl)-lipid Ι₄A about 100 times faster than (Kdo)₂-lipid Ι₄A. The slow, but measurable, rate whereby MsbB acts on (Kdo)₂-lipid Ι₄A may explain why overexpression of MsbB suppresses the temperature-sensitive phenotype of *htrB* mutations. Presumably, the acylxoyacyl group generated by excess MsbB substitutes for the one normally formed by HtrB.

The *htrB* gene was first described by Karow and Georgopoulos (1, 2) as essential for rapid growth of *Escherichia coli* on nutrient broth above 33 °C. At elevated temperatures, peculiar morphological changes are observed in *htrB*-deficient strains, including bulging of the cell surface and filamentation (1–3). At permissive temperatures, *htrB* mutants display increased resistance to bile salts (3). Based on these phenotypes, Karow and Georgopoulos (1, 3–5) suggested function(s) for *htrB* in cell envelope assembly, including possible roles in peptidoglycan, lipopolysaccharide, and fatty acid biosynthesis.

We have recently demonstrated (6) that the *htrB* gene of *E. coli* encodes the lauroyl transferase that acylates the key lipid A biosynthesis intermediate (Kdo)₁₂-lipid Ι₄A (7) (Fig. 1). This conclusion is based on assays of extracts prepared from *htrB*-deficient mutants, in which lauroyl transferase activity is undetectable (6). Conversely, cells overexpressing the *htrB* gene on hybrid plasmids overproduce the transferase several hundredfold (6). We have purified the overproduced enzyme to homogeneity and have shown by N-terminal sequencing that *htrB* is indeed the structural gene for the lauroyl transferase.

Identification of *htrB* as the lauroyl transferase is further supported by fatty acid analyses of lipopolysaccharide isolated from *htrB*-deficient strains of *E. coli*, in which the amount of laurate is reduced (4). Lipid Α from *htrB*-deficient *Hemophilus influenzae* is also under-acylated (8).

Karow and Georgopoulos (3, 5) identified several genes that, when introduced on hybrid plasmids, suppress the temperature-sensitive growth associated with *htrB* mutations. The *msbB* suppressor encodes a putative transport protein with a remarkable similarity to the mammalian *mdr* genes (5). Most *mdr* proteins pump hydrophobic drugs out of animal cells, but some are involved in secretion of phospholipids into bile (9). Although the biochemical function of *msbA* is unknown, the sequence similarity of *msbA* to *mdr* suggests that MsbA might be involved in a translocation process, such as the movement of newly made lipid A from the cytoplasmic surface of the inner membrane to the periplasm (10–12). Whatever its role, the *msbA* gene is essential for growth (5).

A second suppressor gene, designated *msbB*, was found to have 27.5% sequence identity and 42.2% similarity to *htrB* (3) suggesting a biochemical function related to *htrB*. MsbB reverses the temperature sensitivity associated with *htrB* mutations when introduced on plasmids that are maintained at high copy number (3). The *msbB* gene itself is not essential for growth (3). *MsbB* knockout, greatly reduce the amount of myristate attached lipid A, but they do not affect the laurate content (13). Lipopolysaccharide isolated from *msbB* mutants contains penta-acylated lipid A (designated (Kdo)₂-(lauroyl)-lipid Ι₄A in Fig. 1) (13). *MsbB* was discovered independently by Somerville et al. (13), who found that whole *E. coli* cells harboring *msbB* insertions are orders of magnitude less immunostimulatory than are wild-type cells.

Using direct enzymatic assays, we now demonstrate that the *msbB* gene of *E. coli* encodes a distinct, late-functioning acyltransferase of lipid A assembly (7). MsbB functions optimally after laurate incorporation by HtrB has taken place (Fig. 1). The slow, but significant, rate at which MsbB acylates (Kdo)₁₂-lipid Ι₄A explains why *msbB* works only as a high multi-copy suppressor of *htrB* mutations (3). Our findings support the view that at least one acylxyacyl residue must be present on a significant fraction of the lipid A moieties of *E. coli* to allow rapid growth above 33 °C. A preliminary abstract of our findings has appeared (14).

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1 The abbreviation used is: Kdo, 3-deoxy-d-manno-octulosonic acid.
2 S. Carty and C. R. H. Raetz, manuscript in preparation.
**E. coli mscB Gene Encodes an Aeryltransferase**

**Isolation and Preparation of Substrates—**
Plasmid DNAs were isolated using the Wizard miniprep kit (Promega). Other recombinant DNA techniques were performed as described previously (16).

**Recombinant DNA Techniques—**
Plasmid DNAs were isolated using the Wizard miniprep kit (Promega). Other recombinant DNA techniques were performed as described previously (16).

**Plasmids and E. coli K12 strains used in this study**

| Strain                  | Relevant genotype                  | Source          |
|-------------------------|------------------------------------|-----------------|
| W3110                   | Wild-type (htrB\(^{+}\) msbB\(^{-}\)) | E. coli Genetic Stock Center, Yale University |
| XL1-Blue (htrB\(^{+}\) msbB\(^{-}\)) | STRATAGENE       |
| MLK1067 (htrB1::TcCam)  | Ref. 3                            |
| MLK30 W3110 htrB1::TcCam | Ref. 1                            |
| MLK986 (MLK3msbB1::TcCam) | Ref. 3                            |
| pK512 pBluescript carrying htrB\(^{+}\) | Ref. 1            |
| pBS233 pBluescript carrying msbB\(^{-}\) | Ref. 3        |

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| pK512 pBluescript carrying htrB\(^{+}\) | Ref. 1            |
| pBS233 pBluescript carrying msbB\(^{-}\) | Ref. 3        |

**Experimental Procedures**

**Materials—**
ixa, ATP was obtained from DuPont NEN. Pyridine, chloroform, methanol, and 88% formic acid were from Fisher. All detergents were of high quality grade (peroxide- and carbonyl-free). Triton X-100 was from Pierce, and Thesit was from Sigma. Acyl carrier protein was purchased from Sigma. Other items were obtained from the following companies: 0.25-mm glass-backed silica gel 60 thin layer chromatography plates (E. Merck), yeast extract and Tryptone (Difco), and DEAE-Sepharose CL-6B (Pharmacia Biotech Inc.).

**Bacterial Strains and Plasmids—**
Strains used in this study are described previously (6). The MsB-catalyzed acylation of these precursors is 100-fold selective for laurate over myristate (6), consistent with the composition of the acylxoyacyl residues of lipid A isolated from cells (6). The MsB-catalyzed acylation of these precursors is 100-fold selective for the penta-acylated (Kdo)\(^2\)-[4\(^{-}\)\(^{32}\)P]lipid IV\(_A\) generated by HtrB over the tetra-acylated precursor, (Kdo)\(^2\)-lipid IV\(_A\). In extracts, however, MsB is only slightly selective for myristate over laurate. The possibility that HtrB can function on the (Kdo)\(^2\)-[myristoyl]-lipid IV\(_A\) generated at a slow rate by MsB from myristoyl-ACP and (Kdo)\(^2\)-lipid IV\(_A\) has not been examined (as indicated by the question mark []). The proposed structures of the lipids generated by HtrB and MsB are surmised based on what is known about the lipid A structure of E. coli (11, 25–28). Unequivocal analyses to establish the structures of these acylation products of (Kdo)\(^2\)-lipid IV\(_A\) generated in vitro have not yet been carried out.

**Preparation of Cell-free Extracts, Membranes, and Soluble Fractions—**
Crude cell-free extracts were made from 1–2 liters of logarithmically growing cultures. After harvesting by low speed centrifugation at 2 °C, cells were washed once in 30 mM HEPEs, pH 7.5, containing 1 mM EDTA and 1 mM EGTA (half the volume of the original culture). The washed cell pellet was resuspended in 30 mM HEPEs, pH 7.5, containing 1 mM EDTA and 1 mM EGTA (a volume approximately equal to the volume of the cell pellet). Cells were broken using an ice-cold French pressure cell (SLM Instruments, Urbana, IL) at 20,000 psi. The broken cell suspension was adjusted to 10 mM MgSO\(_4\), and DNase I was added to 1 μg/ml. After a brief sonic irradiation on an ice water bath to decrease the viscosity, the suspension was incubated for 30 min at 30 °C. Unbroken cells were removed by centrifugation at 1,000 × g for 10 min. Membranes and soluble fractions were separated by centrifugation at 150,000 × g for 60 min. The supernatant was centrifuged a second time to remove residual contaminating membranes. The membrane pellet was resuspended in 25 ml of 30 mM HEPEs, pH 7.5, containing 1 mM EDTA and 1 mM EGTA, and it was centrifuged again as above to generate the final, washed membrane fraction (~40 mg/ml). The membrane suspension was stored at −80 °C.

Protein concentrations were determined with the bichinonic acid assay (Pierce), using bovine serum albumin as the standard (23).

**Assay for Acyl-ACP-dependent Acylation of Kdo-containing Precursors—**

HtrB-catalyzed acylation was assayed using Method I described earlier (6). In most cases, the reaction mixture contained 50 mM HEPEs, pH 7.5, 0.1% Triton X-100, 25 μM (Kdo)\(^2\)-[4\(^{-}\)\(^{32}\)P]lipid IV\(_A\) (~2 × 10\(^3\) dpm/nmol), 25 μM lauroyl-ACP, 0.1 mg/ml bovine serum albumin, and 0.1–100 μg/ml enzyme at 30 °C in a final volume of 10–20 μl. With highly purified preparations of HtrB, enzyme stability is increased by including 50 mM NaCl and 5 mM MgCl\(_2\) in the assay mixture (6). The MsB-catalyzed acylation of the product generated by HtrB, designated (Kdo)\(^2\)-(lauroyl)-[4\(^{-}\)\(^{32}\)P]lipid IV\(_A\), was assayed in a
reaction mixture containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, and 0.1 mg/ml bovine serum albumin. Unless otherwise indicated, 25 μM (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀₋₉₀ (2 × 10⁶ dpm/nmol) was used as the acceptor, and 25 μM myristoyl-ACP was the donor. Under these conditions, MsbB displays a slight kinetic preference for myristoyl-ACP over lauroyl-ACP. MsbB functions about 100-fold more rapidly with (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀₋₉₀ than with (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀. As the acceptor. Since studies using partially purified MsbB showed a stabilizing effect of sodium chloride, 50 mM NaCl was also included in the reaction in some experiments. MgCl₂ inhibited MsbB slightly. Reactions were stopped by spotting a 4–5-μl sample onto a Silica Gel 60 thin layer chromatography plate. After air drying and developing the thin layer chromatography plate in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v), the plates were exposed to a PhosphorImager screen. The amount of radioactivity present in the labeled spots corresponding to substrate and product were quantitated using a Molecular Dynamics Imager. The specific activity of both HtrB and MsbB was expressed in terms of nmol/min/mg protein.

Analysis of the Lipid A 4′-Monophosphates Extracted from [32P]-Labeled Cells—Cells were grown and labeled by the method of Galloway and Raets (24), with several modifications. Briefly, bacteria from 30°C overnight cultures were inoculated into 20 ml of fresh LB broth and grown for several hours at 30°C until A₆₀₀ had reached 0.1. Next, two 5.0-ml portions of each culture were transferred into two new culture tubes (5 ml each) and grown for 3 h at 30°C, and the other was grown for 3 h at 42°C. [32P]-Labeled cells were washed twice with 5.0 ml of phosphate-buffered saline, pH 7.4, and resuspended in 0.8 ml of the same buffer. Washed cells were extracted at room temperature for 60 min with a single phase Bligh and Dyer mixture, formed by the addition of 2 ml of methanol and 1 ml of chloroform. After centrifugation in a clinical centrifuge at top speed for 20 min, the pellets were recovered and washed once with 5.0 ml of a single phase of Bligh and Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8, v/v). The resuspended pellets were resuspended in 3.6 ml of 0.2 M HCl by sonic irradiation in a bath. The resuspended pellets were incubated at 100°C for 90 min. The acid-hydrolyzed material was converted to a two-phase Bligh and Dyer system by addition of 4 ml of chloroform and 4 ml of methanol. After centrifugation at low speed, the upper phases were removed, and the lower phases were washed twice with 4.0 ml of pre-equilibrated acidic upper phase of a two-phase Bligh and Dyer system, generated by mixing chloroform, methanol, 0.2 M HCl (2:2:1.8, v/v). The washed lower phases were dried under a nitrogen stream. The lipid A 4-mono- and 4′-monophosphates were redissolved in 100 μl of chloroform/methanol (4:1, v/v), and approximately 1000 cpm of the labeled compounds were detected, and the extent of conversion was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Arrows indicate the substrate ([Kdo]₂-laurylethanolamine (4-32P) lipid IV₅₀) and product a ([Kdo]₂-laurylethanolamine (4-32P) lipid IV₅₀) of the reaction.

RESULTS

Activity of the htrB Encoded Laurylethanolamine Transferase of E. coli Is Not Reduced by msbB Insertion Mutations—Extracts of strains bearing htrB mutations do not catalyze lauroyl-ACP-dependent acylation of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀, as shown in Fig. 2 by the absence of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ formation (product a in lane 2). The transfer of laurate from lauroyl-ACP to (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ is efficient in extracts of strains harboring an insertion in msbB (Fig. 2, lane 6), as in the wild type (Fig. 2, lane 4). A similar pattern was observed when myristoyl-ACP was substituted for lauroyl-ACP (not shown), except that the initial rates of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ acylation were 5–10-fold slower with myristoyl-ACP, given the selectivity of HtrB for laurate (6).

Acetylation of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ by Membranes of Wild-type Cells Overexpressing Either htrB or msbB—in crude extracts of membranes preparations from wild-type cells one observes two distinct acylations of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ (lane 7). With lauroyl-ACP as the donor, the first acylation is relatively rapid compared with the second (7). With myristoyl-ACP, the first acylation is 5–10-fold slower than with lauroyl-ACP, but the second acylation is slightly faster than with lauroyl-ACP (7). Given that purified HtrB catalyzes only one rapid acylation of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ (6), it seemed plausible that MsbB might encode the second acyltransferase (Fig. 1) that we previously observed in cell extracts (7).

To approach this problem, we initially incubated membranes of wild-type cells that overexpress either htrB or msbB with ([Kdo]₂-laurylethanolamine (4-32P) lipid IV₅₀ and lauroyl-ACP. As shown in Fig. 3, panel B, overexpression of htrB in the presence of msbB on the chromosome resulted in very rapid formation and accumulation of a monacylated product at the relatively low extract concentrations employed (4 μg/ml). Alternatively, overexpression of msbB in the presence of htrB on the chromosome (Fig. 3, panel A) did not increase the overall extent of ([Kdo]₂-laurylethanolamine (4-32P) lipid IV₅₀ acylation compared with wild-type (hence the need to use 100 μg/ml membranes), but it did result in the accumulation of the diacylated material (product b) at the expense of the monoaoylated product a. The difference between HtrB and MsbB overproduction in a wild-type background is especially apparent when comparing the product distribution at the 40-min time point in panel A of Fig. 3 (excess MsbB) to the 10-min time point in panel B of Fig. 3 (excess HtrB). In both cases the conversion of substrate to product is similar (~30%), but diacylated material is generated in strains overproducing MsbB, whereas monoaoylated product is formed when HtrB is overproduced. The results of Fig. 3 provide support for the idea that MsbB catalyzes the second acylation and can use lauroyl-ACP as the donor under the in vitro assay conditions employed.

Acetylation by MsbB Follows HtrB-catalyzed Acylation of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀—To evaluate further the function of MsbB and its relationship to HtrB, we prepared membranes from strains of msbB or htrB-deficient cells overexpressing htrB and msbB, respectively (6). In this way, we could study cell extracts that contained only one or the other enzyme.

As shown in Fig. 4, panel A, membranes of strain MLK53/pBS233 (containing only msbB) did not catalyze any measurable acylation of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ under the conditions employed. Membranes of cells containing only HtrB (Fig. 4, panel B) catalyzed mainly the formation of product a. In Fig. 4, panel A, the (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ and acyl-ACP were first preincubated for 60 min with 10 μg/ml membranes of MLK53/pBS233. At time 0, a second 60-min incubation was started. Samples were then withdrawn during the second incubation

FIG. 2. Acylation of (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ in cell-free extracts of E. coli W3110, MLK53/htrB, and MLK1067(msbB) . Crude cell-free extracts (1.0 mg/ml) from strain MLK53 (lanes 2 and 3), W3110 (lanes 4 and 5), or MLK1067 (lanes 6 and 7) were incubated together with 25 μM (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ (2 × 10⁶ dpm/nmol) in 50 mM HEPES, pH 7.5, and 0.1% Triton X-100 in a total volume of 20 μl. The reactions of Lanes 1, 2, 4, and 6 also contained 25 μM lauroyl-ACP. The reactions of lanes 3, 5, and 7 contained no added acyl-ACP. No cell extract was added to the reaction in lane 1. After incubation for 30 min at 37°C, 5-μl portions of each reaction mixture were spotted onto a Silica Gel 60 thin layer chromatography plate. The plate was developed in CHCl₃, pyridine, 88% formic acid, water (30:70:18:10, v/v). Radioactively labeled compounds were detected, and the extent of conversion was measured with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Arrows indicate the substrate (Kdo)₂-laurylethanolamine (4-32P) lipid IV₅₀ and product a ([Kdo]₂-laurylethanolamine (4-32P) lipid IV₅₀) of the reaction.
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FIG. 3. Acylation of (Kdo)$_2$-[4$^3$P]lipid IV$_A$ by membranes of the MsbB overproducing strain pBS233/XL1 and of the HtrB overproducing strain pKS12/XL1. Membranes of pBS233/XL1 were used at a protein concentration of 100 µg/ml (panel A). Membranes of pKS12/XL1 were used at a protein concentration of 4 µg/ml (panel B).

Both kinds of membranes were incubated at 37 °C with 25 µM lauroyl-ACP and 25 mM (Kdo)$_2$-[4$^3$P]lipid IV$_A$ (2 × 10$^4$ dpm/nmol) in 50 mM HEPES, pH 7.5, containing 0.1% Triton X-100 in a total volume of 75 µl. At the times indicated, 5 µl of each reaction mixture was spotted onto a Silica Gel 60 thin layer chromatography plate. The plate was developed, and the radioactive spots were detected as in Fig. 2. Arrows indicate the substrate (Kdo)$_2$-[4$^3$P]lipid IV$_A$ and the two predominant acylation products (a and b) that are observed in unfractinated systems.

FIG. 4. MsbB-catalyzed acylation of (Kdo)$_2$-[4$^3$P]lipid IV$_A$ is accelerated by preincubation with HtrB. Membranes (10 µg/ml) of pKS12/MLK1067 (an HtrB over-producer lacking MsbB) were incubated with (Kdo)$_2$-[4$^3$P]lipid IV$_A$ as described in Fig. 3 but with both 25 µM lauroyl-ACP and 25 µM myristoyl-ACP as acyl donors in a total volume of 80 µl. After 60 min of preincubation to allow the HtrB-catalyzed synthesis of (Kdo)$_2$-[lauroyl]-[4$^3$P]lipid IV$_A$, (product a), the reaction was split into two equal portions. To one portion (panel C) membranes (10 µg/ml final concentration) of pBS233/MLK53 (an MsbB overproducer lacking HtrB) were added in 4 µl. To the second portion (panel B) a corresponding volume of buffer was added. The incubation was continued for another 60 min at 37 °C. A third reaction mixture (panel A) with identical concentrations of substrates and buffers, but containing only membranes (10 µg/ml) of pBS233/MLK53 (an MsbB overproducer lacking HtrB), was preincubated and then incubated for another 60 min in parallel. At the times indicated following the preincubation, 5-µl portions of each reaction were spotted onto Silica Gel 60 thin layer chromatography plates. The plates were developed, and radioactive spots were detected as in Fig. 2. Arrows indicate the location of the substrate, (Kdo)$_2$-[4$^3$P]lipid IV$_A$, and the two predominant acylation products (a and b).

period for product analysis at the indicated times. In Fig. 4, panels B and C, membranes of strain MLK1067/pKS12 (containing only htrB$^+$) were preincubated for 60 min with (Kdo)$_2$-[4$^3$P]lipid IV$_A$ and acyl-ACP to generate significant amounts of (Kdo)$_2$-[lauroyl]-[4$^3$P]lipid IV$_A$ (product a). At time 0, only a small volume of buffer was added to the reaction mixture in panel B. In panel C, a 10 µg/ml portion of membranes of MLK53/pBS233 (containing only msbB$^+$) was added at time 0. The incubations were continued for another 60 min and analyzed as in panel A. The system containing only HtrB (panel B) catalyzed the formation of more product a, whereas the system containing both HtrB and MsbB (panel C) resulted in very rapid accumulation of the diacylated product b derived from product a. These findings show that MsbB functions efficiently only after HtrB-catalyzed acylation of (Kdo)$_2$-[4$^3$P]lipid IV$_A$ has taken place (Fig. 4, panel A compared with panel C).

Specific Activity of MsB in Extracts of Mutants and Plasmid-bearing Strains—A direct assay for MsB was developed using 25 µM (Kdo)$_2$-[lauroyl]-[4$^3$P]lipid IV$_A$ as the acceptor and 25 µM acyl-ACP as the donor (see “Experimental Procedures”). Acylation was detected by thin layer chromatography and PhosphorImager analysis as in Fig. 4. With the direct assay, the specific activity of MsB in extracts of wild-type cells was ~0.3 nmol/min/mg, but in extracts of msbB-deficient mutants, the activity was below the limits of detection (~0.03 nmol/min/mg). Overexpression of msb$^+$ on multi-copy hybrid plasmids resulted in ~100-fold higher specific activity of MsB in cell extracts or membranes compared with wild-type, as shown in Table II. The large size of the observed effects on MsB-specific activity supports the proposal that msbB is the structural gene encoding the MsbB acyltransferase.

Solubilization and Partial Purification of MsB—Washed membranes (5 ml) from E. coli MLK53/pBS233 were diluted in 20 mM Tris chloride, pH 7.8, containing 1 mM EDTA and 1 mM EGTA to a final protein concentration of ~17 mg/ml protein (20 mg/ml of protein in a total volume of 12 ml). The membrane suspension was mixed with an equal volume of solubilization buffer, giving final concentrations of 2.5% Triton X-100, 20 mM Tris chloride, pH 7.8, 1 mM EDTA, 1 mM EGTA, 100 mM sodium phosphate, 150 mM NaCl, and 10% glycerol. The solubilization mixture was incubated with slow stirring at 5 °C for 2 h. Insoluble material was removed by centrifugation for 60 min at 170,000 × g at 5 °C. The supernatant was transferred to a new tube and diluted to a final volume of 72 ml with 20 mM Tris chloride, pH 7.8, 1 mM EDTA, and 1 mM EGTA to reduce the NaCl concentration. The solubilized proteins were loaded onto a DEAE-Sepharose CL-6B column (45-ml packed bed-volume) equilibrated in 0.2% Thesit, 20 mM Tris chloride, pH 7.8, 1 mM EDTA, 1 mM EGTA, 50 mM NaCl, and 10% glycerol. After washing the column with 140 ml of equilibration buffer, bound proteins were eluted with a 400-ml linear gradient of 50–500 mM NaCl in 0.2% Thesit, 20 mM Tris chloride, pH 7.8, 1 mM EDTA, 1 mM EGTA, and 10% glycerol. The absorbance at 280 nm of each fraction was measured (Fig. 5, open circles), and the four peak fractions (Fig. 5, shaded area) of MsB activity (filled circles) were pooled. The combined fractions (20 ml) were dialyzed 4-fold in 20 mM Tris chloride, pH 7.8, 1 mM EDTA, and 1 mM EGTA, and the material was loaded onto a 1.8-ml DEAE-Sepharose CL-6B column, equilibrated as above, to concentrate the sample. The total protein was then eluted into a total volume of 1.5 ml of 0.05% Triton X-100, 20 mM Tris chloride, pH 7.8, 1 mM EDTA, 1 mM EGTA, 200 mM NaCl, and 10% glycerol. The fractionated MsB was stored in aliquots at ~80 °C.

Stability and Assay of Purified MsB—Using DEAE-Sepharose-purified MsB, we examined the stability and time dependence of product formation, using enzymatically synthesized (Kdo)$_2$-[lauroyl]-[4$^3$P]lipid IV$_A$ and myristoyl-ACP as
TABLE II

| Purification of MsbB from E. coli MLK53/pBS233 |
|-----------------------------------------------|
| Myristoyl transferase activities were assayed in 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 50 mM NaCl, 25 μM myristoyl-ACP, and 25 mM (Kdo)₂-[lauroyl]-[4-³²P]-lipid IVA (2 × 10⁶ dpm/nmol) at 30 °C. |

| Total volume | Total protein | Specific activity | Yield |
|--------------|---------------|------------------|-------|
| ml           | mg            | nmol × min⁻¹ × mg⁻¹ | %     |
| Membranes    | 5.0           | 208              | 27    | 100  |
| Triton X-100 extract | 24          | 84               | 65    | 96   |
| Triton X-100-insoluble | 5.0         | 90               | 3.6   | 6    |
| DEAE-Sepharose | 1.5         | 5.1              | 640   | 57   |

FIG. 5. Partial purification of solubilized, overproduced MsbB on DEAE-Sepharose CL-6B. Triton X-100-solubilized membrane proteins (84 mg) of strain pBS233/MLK53 were applied onto a 45-ml column of Sepharose CL-6B, as described in the text. Proteins were eluted with a linear gradient consisting of 50–500 mM NaCl. Absorbance at 280 nm (open circles) was used as a measure of total protein in each fraction. MsbB acyltransferase activity (solid circles) was assayed using 5-μl samples of a 1:10 dilution of each fraction. The assay (30 °C) contained 25 μM myristoyl-ACP as acyl donor and 25 μM (Kdo)₂-[lauroyl]-[4-³²P]-lipid IV₆ (2 × 10⁶ dpm/nmol) as lipid acceptor. First shown is the fractionation of MsbB under conditions set approximately two carbons longer than that of HtrB (6). In contrast to the significant activation previously observed with HtrB (6), we found that MsbB was slightly inhibited by the presence of 5 mM MgCl₂ (Fig. 6). However, 50 mM NaCl was beneficial (Fig. 6), as in the case of HtrB (6), and MsbB was stable to preincubation under assay conditions for 30 min in the presence of NaCl (not shown). The MsbB assay carried out in the presence of 50 mM NaCl displayed excellent linearity with respect to time (Fig. 6) and protein concentration (not shown).

Using the improved conditions, the fractions generated in the course of solubilizing and purifying MsbB were reassayed. Table II shows that the final MsbB preparation is purified about 24-fold relative to washed membranes from the overproducing strain employed, and about 2400-fold relative to crude extracts of wild-type cells.

Selectivity of MsbB for Acyl Chain Length—As shown in Fig. 7, DEAE-Sepharose-purified MsbB displays a slight kinetic preference for myristoyl-ACP over lauroyl-ACP. Other acyl donors, including decanoyl-, palmitoyl-, palmitoleoyl-, and (R)-3-hydroxymyristoyl-ACP are inactive as acyl donors when (Kdo)₂-[lauroyl]-[4-³²P]-lipid IV₆ is employed as the acceptor (Fig. 7). The virtual absence of activity with decanoyl-ACP is interesting. HtrB does utilize decanoyl-ACP as a donor in vitro at about one-third of the rate of lauroyl-ACP. Given the fact that we have tested the same acyl-ACP preparations with both HtrB (6) and MsbB (Fig. 7), we conclude that the optimal activity of MsbB with various acyl-ACPs under our assay conditions is set approximately two carbons longer than that of HtrB. In living cells, however, the selectivity of HtrB for laurate and of MsbB for myristate must be higher. No decanoate is found in the position normally occupied by laurate on lipid A under ordinary growth conditions in living cells (Fig. 1), and very little laurate is incorporated in place of myristate (11, 25–28).

Selectivity of MsbB for Acylated Lipid A Precursors—The DEAE-Sepharose-purified preparation of MsbB was used to confirm the specificity of the enzyme for the penta-acylated acceptor, first suggested by the results of Fig. 4. As shown in Fig. 8, we compared (Kdo)₂-[4-³²P]-lipid IV₆, (Kdo)₂-[lauroyl]-[4-³²P]-lipid IV₆, and (Kdo)₂-[lauroyl]-[4-³²P]-lipid IV₆ (each 25 mM) with 50 mM NaCl and 5 mM MgCl₂ were added to the reaction; open squares, reaction with 5 mM MgCl₂ only; open triangles, reaction with 50 mM NaCl only.

FIG. 6. Effects of MgCl₂ and NaCl on msbB-encoded myristoyltransferase activity. DEAE-Sepharose CL-6B purified MsbB was assayed at a protein concentration of 0.1 μg/ml in 20-μl reaction mixtures containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 25 μM myristoyl-ACP, and 25 μM (Kdo)₂-[lauroyl]-[4-³²P]-lipid IV₆ (2 × 10⁶ dpm/nmol). Open circles, 50 mM NaCl and 5 mM MgCl₂ were added to the reaction; open squares, reaction with 5 mM MgCl₂ only; open triangles, reaction with 50 mM NaCl only.
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The initial rates of acylation of 25 μM (Kdo)₂[(lauroyl)-14C]-lipid IVₐ (2 × 10⁴ dpm/nmol) were determined at 30 °C using different acyl-acyl carrier proteins as donors (all at 25 μM). In addition to the indicated substrates, the transferase reaction mixtures contained DEAE-Sepharose CL-6B purified MsB at a concentration of 0.1 μg/ml, 50 mM HEPES, pH 7.5, 0.1% Triton X-100, and 50 mM NaCl. Reactions (20 μl) were incubated at 30 °C. The initial rates of (Kdo)₂[(lauroyl)-14C]-lipid IVₐ acylation were normalized to that observed using different acyl-acyl carrier proteins as donors (all at 25 μM). The other acyl chains tested were 10:0, 12:0, laurate; 16:0, palmitate; 16:1c, palmitoleate; 14:0, 12:0, decanoate; 10:0, myristate; 10:1c, hydroxymyristate; 16:0, myristoleate; 18:0, stearate; 16:1c, 18:0, oleyl; 18:1, oleate; 18:2, linoleate; 18:3, linolenate; and 20:4, arachidonate (Fig. 1). In reactions (20 μl) were incubated at 30 °C. The initial rates of (Kdo)₂[(lauroyl)-14C]-lipid IVₐ acylation were normalized to that observed with myristoyl-acyl carrier protein (4:0:0:0) as the acyl donor. The other acyl chains tested were 10:0, 12:0, laurate; 16:0, palmitate; 16:1c, palmitoleate.

FIG. 7. Acyl chain length specificity of the msbB-encoded myristoyltransferase. The initial rates of acylation of 25 μM (Kdo)₂[(lauroyl)-14C]-lipid IVₐ (2 × 10⁴ dpm/nmol) were determined at 30 °C using different acyl-acyl carrier proteins as donors (all at 25 μM). In addition to the indicated substrates, the transferase reaction mixtures contained DEAE-Sepharose CL-6B purified MsB at a concentration of 0.1 μg/ml, 50 mM HEPES, pH 7.5, 0.1% Triton X-100, and 50 mM NaCl. Reactions (20 μl) were incubated at 30 °C. The initial rates of (Kdo)₂[(lauroyl)-14C]-lipid IVₐ acylation were normalized to that observed using different acyl-acyl carrier proteins as donors (all at 25 μM). The other acyl chains tested were 10:0, 12:0, laurate; 16:0, palmitate; 16:1c, palmitoleate. 

Our procedure in Fig. 8. Lipid acceptor specificity of the msbB-encoded acyltransferase. DEAE-Sepharose CL-6B purified MsB was used at 0.1 μg/ml in reaction mixtures containing 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 50 mM NaCl, and 2 μM of one of the following acceptor lipids: lanes 1 and 2, [4-32P]lipid IVₐ (2 × 10⁴ dpm/nmol); lanes 3 and 4, (Kdo)₂-[4-32P]lipid IVₐ (1 × 10⁵ dpm/nmol); lanes 5 and 6, (Kdo)₂[(lauroyl)-14C]-lipid IVₐ (2 × 10³ dpm/nmol). The reactions (10 μl) were incubated at 30 °C for 60 min either with (lanes 1, 3, and 5) or without (lanes 2, 4, and 6) 25 μM myristoyl-ACP. Arrows indicate migration of lipid substrates and generated products, a, (Kdo)₂[(myristoyl)-14C]-lipid IVₐ and b, (Kdo)₂-[4-32P]lipid IVₐ. Proposed structures are shown in Fig. 1.

due is treated with 0.2 M HCl at 100 °C to release all the lipid A moieties of lipopolysaccharide as a series of 4'-monophosphates (24). These compounds are separated into hexa-acylated, penta-acylated, and tetra-acylated species, using silica gel thin layer chromatography (24), and they can be detected by autoradiography or PhosphorImage analysis.

As shown in Fig. 9 (lanes 2 and 3), the lipid A-derived 4'-monophosphates of wild-type cells, labeled for 3 h at either 30 or 42 °C in LB broth (15) with [32P]₀, consist mostly of hexa-acylated, some penta-acylated, and a few tetra-acylated forms. The relative abundance of the hexa-acylated species may be underestimated slightly by our procedure because of partial deacylation caused by the acid hydrolysis (24). However, strains defective in msbB yield mostly penta-acylated 4'-monophosphates with only minor amounts of the tetra-acylated species at both 30 or 42 °C (Fig. 9, lanes 4 and 5), consistent with the proposed function of MsB (Fig. 1). Under nonpermissive conditions (42 °C), strains defective in htrB or in both htrB and msbB (Fig. 8, lanes 7 and 9, respectively) yield mostly tetraacylated lipid A 4'-monophosphates. Interestingly, when grown at 30 °C, these mutants do generate significant amounts of penta-acylated and even some hexa-acylated forms (Fig. 9, lanes 6 and 8), the origin of which is not entirely clear (see “Discussion”). However, when msbB+ is overexpressed in htrB-deficient cells, growth and considerable amounts of additional penta-acylated 4'-monophosphates are restored at 42 °C (Fig. 9, lane 11 versus lane 7). These observations support the view

FIG. 9. Extent of lipid A acylation in wild-type and mutant strains determined by [32P] labeling. Cells of each of the indicated strains were grown at 30 °C on LB broth until A₆₀₀ had reached -0.1. The cultures were divided into two 5-ml portions, and [32P]₀ (5 μCi/ml) was added to both. One culture was grown for 3 h at 30 °C, and the other was grown for 3 h at 42 °C. After this period to allow for uniform labeling of the cellular lipids, the lipid A 4'-monophosphate fractions were isolated by a modification of the procedure of Galloway and Raetz (24). Approximately 1000 cpm of isolated lipid A [32P-4] 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 (50:50:16:5, v/v). The plate was then dried and exposed overnight to a PhosphorImager screen. The positions of the hexa-acylated, penta-acylated, and tetra-acylated [32P-4] monophosphates, as well as the origin, are indicated. Lanes 1 and 12 show the position of a tetra-acylated [32P-4] monophosphate standard that was generated by acid hydrolysis of [4-32P]lipid IVₐ. Even lanes 4 and 5, MLK1067; lanes 6 and 7, MLK53; lanes 8 and 9, MLK986; lanes 10 and 11, MLK53/pBS233.
that high level overexpression of MsbB bypasses the need for HtrB action prior to MsbB-catalyzed acylation. Rapid cell growth apparently requires that a critical fraction of the lipid A moieties of lipopolysaccharide be at least penta-acylated. The enzymatic source of these acylations seems not to be as important.

**DISCUSSION**

As shown by the results of Figs. 2–4, 8, and 9, msbB encodes a distinct acyltransferase of lipid A assembly that functions optimally after laurate incorporation by HtrB has occurred. The proposed intermediates generated by MsbB and HtrB are shown in Fig. 1. These structural proposals are based on what is known about lipid A released from *E. coli* lipopolysaccharide by acid hydrolysis (11, 25–28). The proposed structures also are in accord with the molecular weights of (Kdo)_2-lipid IV_A acylation products generated previously with crude cell extracts (7). The lauroyl and myristoyl groups are attached by HtrB and MsbB to the lipid IV_A moieties of (Kdo)_2-lipid IV_A (7). However, the structures of the acylated derivatives of (Kdo)_2-lipid IV_A generated using homogeneous preparations of HtrB and MsbB remain to be established unequivocally.

Besides HtrB and MsbB, there are at least two additional enzymes that may be able to acylate (Kdo)_2-lipid IV_A in *E. coli* extracts. One of these transfers palmitate from the 1-position of a glycerolphospholipid to the (R)-3-hydroxy moiety of the N-linked (R)-3-hydroxymyristate of the proximal glucosamine unit (20). The palmitoyltransferase has not been purified, and the gene encoding it is unknown. In addition, we have recently obtained evidence for a cold shock-induced enzyme that transfers palmitoleate from palmitoleoyl-ACP to (Kdo)_2-lipid IVA.3

E. coli mutants defective in htrB or in both htrB and msbB contain lipid A moieties that are mainly tetra-acylated when cells are grown at 42 °C for 3 h. The presence of excess msbB+ on hybrid plasmids maintained at high copy number restores some penta-acylated lipid A species (Fig. 9) and suppresses the temperature-sensitive growth phenotype associated with htrB mutations (3). These findings suggest that the slow rate of acylation of (Kdo)_2-lipid IV_A catalyzed by MsbB (Fig. 8, product a') may be sufficient to generate a significant number of acylxoyacyl moieties when MsbB is overproduced in htrB-deficient cells. We presume that (Kdo)_2-(myristoyl)-lipid IV_A, having the proposed structure shown at the bottom of Fig. 1, is being generated when the htrB mutation is suppressed by overexpression of msbB+

*E. coli* mutants defective in htrB or in both htrB and msbB do contain some penta- and hexa-acylated lipid A species when grown at 30 °C (Fig. 9). These species might arise by action of the palmitoyltransferase (20) or the cold-induced palmitoleoyltransferase noted above. To determine the origin of these more highly acylated lipid A species present in mutants defective in both htrB and msbB, it will be necessary to carry out additional chemical analyses. Preliminary results with laser desorption mass spectrometry4 indicate that lipid A isolated from mutants defective in both htrB and msbB grown at 30 °C consists of a mixture of tetra-acylated and penta-acylated forms. Both forms contain four hydroxymyristoyl chains, but the penta-acylated species contains an additional palmitate or palmitoleate.

The effects of the *msbA* multi-copy suppressor (5) on the extent of acylation of the lipid A moieties present in mutants defective in both htrB and msbB remain to be examined. Recently, Polissi and Georgopoulos (31) have found that lipopolysaccharide accumulates in the inner membranes of htrB−deficient mutants. Introduction of both msbA+ and orfE' (an essential downstream gene of unknown function) on hybrid plasmids partially restored-deficient translocation of lipopolysaccharide to the outer membrane in htrB mutants (31). Insertion mutations that conditionally inactivate both msbA and orfE also appear to accumulate lipopolysaccharide in their inner membranes (31). If msbA is really involved in lipid A export, as anticipated because of its sequence similarity to mammalian Mdr proteins, one should see no restoration of lipid A acylation upon suppression of htrB−msbB− mutants by msbA+. If msbA, like msbB, encodes a distinct acyltransferase, however, then the extent of lipid A acylation would be increased when mutants defective in both htrB and msbB are complemented by msbB.

A search of genome data bases indicates that *H. influenzae* contains htrB and msbB homologues (8). Based on mass spectrometry of lipid A isolated from htrB mutants of *H. influenzae* (31), it is likely that HtrB function is conserved in this organism. All known HtrB and MsbB sequences contain the peptide LCFP in the vicinity of residue 70.

The identification of the genes that are responsible for the early and late acylations of lipid A should facilitate the construction of *E. coli* strains possessing greatly reduced or altered endotoxin activities. The msbB insertion mutations described by Somerville et al. (13) reduce the ability of intact *E. coli* to activate macrophages by several orders of magnitude. By further modifying the structure and composition of *E. coli* lipid A, it may be possible to create strains with virtually no endotoxin activity. For instance, substitution of *E. coli* lipid A by *Pseudomonas* lipid A (32), coupled with deletion of msbB, would result in the generation of a lipid A with considerable similarity to that of *Rhodobacter sphaeroides* (33, 34). Lipid A of *R. sphaeroides* actually antagonizes the activities of wild-type *E. coli* endotoxin (33, 34). Such *E. coli* mutants, if viable, might be very useful for the expression and preparation of proteins intended for injection into animals, since quantitative removal of lipopolysaccharide would not be as critical. Modification of the structure of lipid A in living bacterial cells might also provide new insights into the functions of lipid A in outer membrane biogenesis and during infection of animals.

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Function of the *Escherichia coli msbB* Gene, a Multicopy Suppressor of *htrB* Knockouts, in the Acylation of Lipid A: ACYLATION BY MsbB FOLLOWS LAURATE INCORPORATION BY HtrB

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