Function of the htrB High Temperature Requirement Gene of Escherichia coli in the Acylation of Lipid A

HtrB CATALYZED INCORPORATION OF LAURATE

(Received for publication, February 8, 1996)

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By assaying lysates of Escherichia coli generated with the hybrid λ bacteriophages of an ordered library (Kohara, Y., Akiyama, K., and Isono, K. (1987) Cell 50, 495-508), we identified two clones (λ232 and λ233) capable of overexpressing the lauryl transferase that functions after 3-deoxy-o-manno-octulosonic acid (Kdo) addition in lipid A biosynthesis (Brozek, K. A., and Raetz, C. R. H. (1990) J. Biol. Chem. 265, 15410-15417). The E. coli DNA inserts in λ232 and λ233 suggested that a known gene (htrB) required for rapid growth above 33 °C might encode the lauryl transferase. Using the intermediate (Kdo)₂-lipid IVA as the laurate acceptor, extracts of strains with transposon insertions in htrB were found to contain no lauryl transferase activity. Cells harboring hybrid htrB+ plasmids overproduced transferase activity 100-200-fold. The overproduced transferase was solublized with a non-ionic detergent and purified further by DEAE-Sepharose chromatography. With lauroyl acyl carrier protein as the donor, the purified enzyme rapidly incorporated one laurate residue into (Kdo)₂-lipid IVA. The rate of laurate incorporation was reduced by several orders of magnitude when either one or both Kdos were absent in the acceptor. With a matched set of acyl-acyl carrier proteins, the enzyme incorporated laurate 3-8 times faster than decanoate or myristate, respectively. Transfer of palmitate, palmitoleate, or 3-hydroxymyristate was very slow. Taken together with previous studies, our findings indicate that htrB encodes a key, late functioning acyltransferase of lipid A biosynthesis.

Lipid A, the hydrophobic anchor of the outer membrane lipopolysaccharide of Escherichia coli (1-5), consists of a glucosamine disaccharide that is phosphorylated at positions 1 and 4', and is acylated with R-3-hydroxyacylmyristate at positions 2, 3, 2', and 3' (Fig. 1). Lipid A of wild-type E. coli cells contains two additional fatty acyl chains, primarily laurate and myristate (3, 6-8). The latter are esterified to the R-3-hydroxy groups of the distal glucosamine residue (Fig. 1), forming the acyloxyacyl moieties that are characteristic of lipid A (1, 3, 6-8). Variations in the composition and location of the non-hydroxylated acyl chains can occur (1, 8). For instance, minor lipid A species are observed in E. coli in which the myristate residue is missing entirely (8) or is replaced with palmitate (not shown in Fig. 1) (9). An additional palmitate residue may also sometimes be present in acyloxyacyl linkage on the proximal glucosamine unit (not shown in Fig. 1), either with or without the myristate on the distal glucosamine (1, 4, 8). The laurate residue that is attached to the distal N-linked R-3-hydroxyacylmyristate (Fig. 1) is found in almost every lipid A moiety of wild-type cells (8, 9).

In previous studies, we described the existence of novel acyltransferases in extracts of E. coli that can incorporate one or two laurate (or myristate) residues into lipid A precursors (10). The lauroyl/myristoyl transferases require lauroyl- or myristoyl acyl carrier proteins (ACP) as donors (10). They appear to function at a late stage of lipid A assembly, since they recognize lipid A disaccharide precursors that are glycosylated with Kdo as substrates (Fig. 1) (10). Their extraordinary Kdo dependence explains why lipid IVA (Fig. 1), rather than fully acylated lipid A, accumulates in living cells when the biosynthesis of Kdo is interrupted (11-14).

Prior to the present work, no late acyltransferase had been purified (10), and gene(s) encoding these enzymes were not known (3). The issue of whether one or more distinct acyltransferases were required for acyloxyacyl group formation was not resolved (10). A priori, it seemed reasonable that a separate enzyme would be needed for the generation of each acyloxyacyl moiety. In cell extracts, two laurates, two myristates, or one of each can be transferred efficiently to (Kdo)₂-lipid IVA (10), despite the distinct composition of the dominant lipid A molecular species made in wild-type cells (Fig. 1) (7, 8).

To identify a gene encoding a Kdo-dependent late acyltransferase, we have assayed individual E. coli lysates generated with each of the hybrid λ bacteriophages of the Kohara library (15, 16) for overproduction of the laurayl transferase that acts on (Kdo)₂-lipid IVA (Fig. 1) (10). In this manner, we have discovered that a known gene (htrB), described by Karow and Georgopoulos (17-21) as required for growth on rich media above 33 °C, encodes the lauryl transferase (Fig. 1). This finding explains why lipid A isolated from htrB deficient mutants contains very little laurate (20). The msbB gene (19), a high multi-copy suppressor of the temperature sensitivity associated with htrB mutations, encodes a separate late acyltransferase with a strong kinetic preference for the penta-acylated lipid generated (Fig. 1) by HtrB (22). The envelope-related alterations associated with mutations in the htrB gene (17, 19), such as bulging of the cell surface and deoxycholate resistance, can now be explored in a biochemical context. A preliminary communication of our results has appeared in abstract form (22).

EXPERIMENTAL PROCEDURES

Materials—[γ-³²P]ATP was obtained from DuPont NEN. Pyridine, chloroform, methanol, and 88% formic acid were from Fisher. All other reagents were of the highest grade available.

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*This research was supported by National Institutes of Health Grant GM-51310 (to C. R. H. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: ACP, acyl carrier protein; bis-tris, 2-[bis(2-hydroxyethyl)amino]2-(hydroxyethyl)-propane-1,3-diol.
tergents were of high quality grade (peroxide and carbonyl free). Triton X-100 was Surfact-Amps grade from Pierce, and Thesit was from Boehringer Mannheim. 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-Phosphor-SEpharose CL-6B, Pharmacia.

Bacterial Strains and Plasmids—Strains used in this study are derivatives of E. coli K12, and their genotypes are listed in Table I. Cultures were grown in Luria broth, consisting of 5 g of NaCl, 5 g of yeast extract, and 10 g of tryptone/liter (23). Antibiotics were added, when required, at 100 μg/ml for ampicillin, 12 μg/ml for tetracycline, and 10 μg/ml for chloramphenicol.

Recombinant DNA Techniques—Plasmid DNAs were isolated using the Wizard miniprep kit (Promega). Other recombinant DNA techniques were performed as described previously (24).

Screening of the Kohara Library for Overproduction of Lauroyl Transferase Activity—A set of fresh lysates was created from a copy of the original Kohara library (15, 16). A culture of the host, E. coli W3110, grown to saturation overnight in LB broth supplemented with 10 mM MgSO4 and 0.2% maltose, was mixed with an equal volume of 10 mM MgCl2 and 10 mM CaSO4. Using several 96-well microplate units, 10-μl portions of the host cell suspension were mixed with 5 μl of each of the 476 lysates of the Kohara library (15, 16). After allowing 15 min for infection, 150-μl portions of LB broth supplemented with 10 mM MgSO4 were added to each well. The lysates from the original library were used undiluted or diluted 1/10, as required, to accomplish lysis of the W3110 host within 5–8 h after infection. The microtitre plates were incubated on a shaker at 37°C. At 4.5 h after infection, and at 30-min intervals thereafter, the plates were inspected for wells displaying complete lysis. As soon as cultures showed lysis, the lysates were transferred to a new microtitre plate and stored at 4°C, while the unlysed cultures in the original microtitre plate were incubated further at 37°C. At the end of the experiment, the lysates were all stored at −80°C.

For the purpose of screening the entire library, lysates (0.5 μl of a 1:5 dilution) were assayed for lauroyl transferase activity. In a 10-μl screening assay mixture containing 50 mM Hepes pH 7.5, 0.1% Triton X-100, 10 μM (Kdo)2-[4-32P]lipid IVA (1 × 106 dpm/μl), 25 μM lauroyl-ACP, and 0.2 mg/ml bovine serum albumin, reactions were incubated for 10 min at 30°C. A 5-μl portion of each reaction mixture was spotted onto a Silica Gel 60 thin layer chromatography plate, which was developed and subjected to Phosphorimager analysis to determine the extent of acylation catalyzed by each extract (see below).

Assay for Lauroyl-ACP-dependent Acylation of (Kdo)2-lipid IVA—To determine the specific activity of the lauroyl transferase in various extracts and purified fractions, assays were generally performed at 30°C, as described previously (10). The standard reaction mixture (Method I) contained 50 mM Hepes pH 7.5, 0.1% Triton X-100, 25 μM (Kdo)2-[4-32P]lipid IVA (2 × 106 dpm/μl), 25 μM lauroyl-ACP, 0.2 mg/ml bovine serum albumin, and 0.4–1000 μg/ml enzyme protein, as indicated, in a final volume of 20 μl. Reactions were stopped by spotting a 4–5-μl portion onto a Silica Gel 60 thin layer chromatography plate. After a few minutes to allow the spots to dry, the plates were developed in chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v/v), and the radioactivity was determined using a Molecular Dynamics Phosphorimager. The specific activity of the lauroyl transferase was expressed in terms of nanomoles/min/mg of protein.

In the course of purifying the lauroyl transferase, an improved assay was developed (Method II). The latter was identical to Method I, except that 5 mM MgCl2 and 50 mM NaCl were included in the reaction mixture to stabilize the enzyme.

Preparation of Cell-free Extracts, Membranes, and Soluble Fractions for Analysis of the Lauroyl Transferase—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 a French Press (SLM Instruments, IL) at 20,000 psi. The broken cell suspension was adjusted to 10 mM MgSO4 and DNase I was added to 1 μg/ml. After a brief sonication 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 1000 × 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.

Protein concentrations were determined with the bichinchoninic assay (Pierce), using bovine serum albumin as the standard (25).

Isolation and Preparation of Substrates—Lipid IVA (13), (Kdo)2-lipid IVA (26, 27), (4-32P)lipid IVA (28, 29), and (Kdo)2-[4-32P]lipid IVA (26, 27) were prepared as described previously. A derivative of (4-32P)lipid IVA (designated Kdo2-[4-32P]lipid IVA) was synthesized from (4-32P)lipid IVA under the same conditions used to make (Kdo)2-[4-32P]lipid IVA, except that membranes (1 mg/ml final concentration) isolated from Haemophilus influenzae (30) were substituted for the purified E. coli Kdo transferase (27).

Various acyl-ACPs were synthesized from the corresponding fatty acids and commercial acyl carrier protein, as described previously (31), except that the immobilized acyl-ACP synthase was replaced with 80 μg/ml solubilized membrane protein from the acyl-ACP synthase over-producing strain, E. coli LCH109 (LPS/GP1–2) (29).

To obtain the acyl-ACP synthetase, LCH109/LPS/GP1–2 membrane solubilized using a modification of the procedure of Rock and Cronan (32, 33). Two 1-ml cultures of LCH109/LPS/GP1–2 were grown at 30°C until the cultures reached an A600 of approximately 0.4. The cultures were then shifted to 42°C for 30 min. Next, the cultures were allowed to continue growing for 90 min at 37°C. To harvest, the cells were centrifuged at 4500 × g for 10 min and resuspended in 15 ml of 50 mM Tris-HCl pH 8.0. A French pressure cell at 18,000 p.s.i. was used to disrupt the cells, and unbroken cells were removed by centrifuging at 1000 × g for 10 min. The supernatant was made 10 mM in MgCl2 by adding 1 mM MgCl2. Then, the extract was centrifuged for 1 h at 150,000 × g. The pellet was resuspended in 5 ml of 50 mM Tris-HCl, pH 8.0. Next, a 5-ml solution of 50 mM Tris-HCl, pH 8.0, containing 4% Triton X-100 and 20 mM MgCl2 was added to solubilize the inner membrane fraction. The suspension was incubated on ice for 30 min. The solubilized membranes were centrifuged a second time at 150,000 × g for 1 h to remove outer membrane proteins, which were not extracted efficiently under these conditions. The supernatant was stored in aliquots at −80°C.

The enzymatic acylation of ACP with laurate, myristate, palmitate, decanoate, palmitoleate, or 3-hydroxyacylside was carried out as follows. ACP (1 mg) and 8.6 ml di-thiothreitol were incubated in 500 μl of 40 mM Tris-HCl, pH 8.0, in a sealed tube at 37°C for 1 h. Next, a 320-μl solution consisting of 0.7 × Licitin 40 mM MgCl2, 20 mM ATP, pH 8.0, 750 μM fatty acid, 2.7% Triton X-100, and 540 mM Tris-HCl, pH 8.0, was added to the tube with the ACP. Last, 400 μl of 0.25 mg/ml LCH109/LPS/GP1–2 solubilized membranes was added, and the acylation reaction was allowed to proceed at room temperature for 1–2 h. The extent of acylation was determined by analyzing 5-μl portions of the reaction mixture on a polyacrylamide gel electrophoresis gel system (34). To isolate the product, the reaction mixture was diluted 10-fold with water and loaded onto a 1-ml column of DEAE-Phosphor-Sepharose equilibrated with 10 mM bis-tris, pH 6.0. The column was washed with 5 bed volumes of 10 mM bis-tris, pH 6.0, 5 volumes of 10 mM bis-tris, pH 6.0, containing 50% isopropanol, and 5 volumes of 10 mM bis-tris, pH 6.0. The column was eluted with 3 volumes of 10 mM bis-tris, pH 6.0, containing 0.2 M LiCl and 3 volumes of 10 mM bis-tris, pH 6.0, containing 0.6 M LiCl. Fractions of 1 ml were collected. The acyl-ACPs eluted in the second 0.6 M LiCl fraction were concentrated and exchanged into distilled H2O using a Centricon-3 membrane (Amicon). The acyl-ACPs were about 90% pure, as judged by electrophoresis in the polyacrylamide/urea gel system and staining with Coomassie Blue (34).
RESULTS

Preparation of Kohara Library λ Lysates on E. coli W3110—Lysates were generated in E. coli strain W3110, using an ordered subset of the E. coli chromosomal λ library of Kohara (15, 16), as described under “Experimental Procedures.” Every insert in this “miniset” collection has been mapped with restriction enzymes and positioned on the E. coli chromosome (15, 16), facilitating the identification of genes. Satisfactory lysates were obtained with 461 of the 476 clones that constitute the miniset collection. The remaining 15 clones did not cause lysis of E. coli W3110, probably because of a low titer in the original library lysates. No attempt was made to retrieve these 15 clones.

Assaying Enzymes of Lipid Biosynthesis in the λ Lysates—Before screening all the lysates for overproduction of the lauroyl transferase, the ability of these lysates to overexpress a characterized enzyme of lipid A biosynthesis with a known structural gene was validated. The kdtA gene, encoding Kdo transferase (Fig. 1), was previously mapped to the overlapping DNA segments present in λ clones [573]4D2 and [572]2A6 of the Kohara library (16, 35). The λ lysates generated with these clones on W3110 displayed a 5–10-fold higher specific activity of Kdo transferase than lysates generated from other, randomly selected λ clones (data not shown). Overexpression, like that seen with kdtA, was also observed in lysates generated with clones harboring two other mapped genes of lipid biosynthesis (dgk and glpK) (16). These findings demonstrate that certain cloned E. coli genes present on non-lysogenic hybrid λ vectors are transcribed and translated efficiently during infection. It is possible to detect clones containing a gene of interest by assaying for overexpression of enzymatic activity above the background derived from the chromosomally encoded gene. Screening for overexpression of enzyme activity in lysates prepared with hybrid λ libraries therefore affords a useful alternative for identifying novel genes in such libraries, especially if no sequence information is available.

Screening for E. coli DNA Fragments Encoding the Lauroyl Transferase—Given the results with the Kdo transferase, all 461 lysates generated in E. coli strain W3110 with the Kohara library were assayed for overproduction of the lauroyl transferase that acts on (Kdo)2-[^4-^32P]lipid IVa (Fig. 1) (10). Appropriate dilutions of these lysates were used to give a conversion within the linear range of the assay (1–10% under these conditions) for the chromosomally encoded activity, as described under “Experimental Procedures.” Screening the lysates in this manner resulted in the identification of two clones (Fig. 2) that overexpressed the lauroyl transferase, as judged by thin layer chromatography and PhosphorImager analysis. Lysates from clones [232]1H7 and [233]E4H10S, containing adjacent and partially overlapping chromosomal fragments, catalyzed 25 and 45% conversion of substrate to product, respectively, as compared to 5.5 and 7.1% for the two flanking clones, [231]1C7 and [234]E3G11 (Fig. 2). The overexpressed transferase displayed an absolute dependence on the addition of lauroyl-ACP as a cosubstrate (Fig. 2), as did the transferase present in extracts of control cells.

Genes Present on [232]1H7 and [233]E4H10S—The cloned DNA fragments in [232]1H7 and [233]E4H10S include approximately 11 kilobases of overlapping sequences, according to the EcoMap6 (16). Since both these clones directed overexpression of the transferase, we assumed that the entire gene encoding the transferase should reside within the overlap. A few sequenced genes had previously been mapped to this region (near minute 25), one of which is htrB (17, 18). Strain MLK53, in which htrB is disrupted by Tn10 insertion (17, 19), is unable to grow above 33 °C on rich media. MLK53 also is resistant to four...
times the concentration of deoxycholate as is wild-type E. coli, and addition of low levels of cationic detergents to the medium suppresses the temperature-sensitive growth associated with htrB mutations (19). Since these properties, in conjunction with morphological alterations observed in MLK53 (17, 19), are indicative of changes in the outer membrane, htrB seemed to be a plausible candidate for a gene involved in lipid A biosynthesis. Although the htrB gene had been cloned and sequenced (17, 18), no enzymatic function had been ascribed to its product.

The htrB Gene Encodes a Lauroyl Transferase Involved in Lipid A Biosynthesis—As shown in Fig. 3, crude cell free extracts prepared from E. coli MLK53 (17, 19), a strain in which the htrB gene is inactivated by insertion of Tn10, contained no detectable lauroyl transferase activity. Membranes of MLK53 also were devoid of lauroyl transferase (Table II). Wild-type E. coli host strains transformed with the multi-copy plasmid pKS12 (17), harboring htrB, also were devoid of lauroyl transferase (Table II). The pKS12 expressed lauroyl transferase activity, like the wild-type, was located primarily in the membrane fraction. The observed membrane localization of the enzyme is consistent with the hydrophathy profile of htrB (18, 19). The large effects of htrB mutations or htrB overexpression on lauroyl transferase specific activity (Table II) strongly suggest that htrB is the structural gene for the transferase.

Karow and Georgopoulos (19) identified and isolated another gene of unknown function, designated msbB, that displays significant sequence homology to htrB. When present on hybrid plasmids maintained at very high copy numbers, msbB suppressed the temperature-sensitive growth phenotype of MLK53 (19). Unlike htrB, however, msbB is not essential for growth at any temperature (19). Membranes of strain MLK1067 (19), harboring an insertion in msbB, contain normal amounts of lauroyl transferase (Table II). Overexpression of msbB on hybrid plasmids does not result in increased levels of lauroyl transferase (data not shown). When pKS12 (htrB+) is transformed into E. coli MLK1067 (msbB::tacam) (19), lauroyl transferase activity is overproduced, just as when pKS12 is introduced into wild-type strains, like XL1-Blue (Table II). These findings are consistent with the hypothesis that htrB and msbB encode distinct enzymes, and are not likely to be regulatory genes affecting the same enzyme.

Myristoyl-ACP could replace lauroyl-ACP as the acyl donor in acylations catalyzed by crude extracts or isolated membranes from E. coli W3110, XL1-Blue, or MLK1067/pKS12 (data not shown). However, the reaction rate with myristoyl-ACP as the donor and (Kdo)2-[32P]lipid IVα as the acceptor was 5–10 fold slower than with lauroyl-ACP. Extracts of MLK53 were unable to acylate (Kdo)2-[32P]lipid IVα when myristoyl-ACP was employed as the substrate (data not shown), as observed with lauroyl-ACP (Fig. 3 and Table II).

Solubilization of Lauroyl Transferase from Membranes of Overproducing Strains and Fractionation on DEAE-Sepharose—The lauroyl transferase was solubilized in an active form from membranes of E. coli MLK1067/pKS12, using the Triton X-100 analog, Triton X-100 analog, Thesit. Membranes (3.8 ml at 18 mg/ml protein) were incubated for 2 h at 4 °C with an equal volume of solubilization buffer, yielding final concentrations of 2.5% Triton X-100 analog, 1 M Tris chloride, pH 7.8, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10% glycerol, and 100 mM potassium phosphate. Insoluble material was removed by centrifugation for 60 min at 170,000 × g. These conditions solubilized 98% of the lauroyl transferase activity, while only releasing about 30% of the total membrane proteins, giving a 3-fold purification before DEAE-fractionation (Table III). The solubilized protein (20 mg) was diluted 4-fold with buffer consisting of 20 mM Tris chloride, pH 7.8, 1 mM EDTA, 1 mM EGTA, and 10% glycerol, and the
sample was applied onto a 45-ml column of DEAE-Sepharose CL-6B, equilibrated with 20 mM Tris chloride, pH 7.8, 0.2% Triton X-100 (w/v). At 5 mM MgCl2, the Triton X-100 dropped off, presumably because of surface dilution of the substrate present. At higher Triton X-100 concentrations, the activity of lauroyl transferase functions at least 2 orders of magnitude lower than that of lauroyl-ACP in cells. In crude extracts, lauroyl-ACP is a more efficient acyl donor than myristoyl ACP (see above). To determine the acyl chain specificity of the purified lauroyl transferase, acyl-ACPs were not very effective substrates (Fig. 7). The fact that decanoate is not present in lipid A isolated from living cells (8) suggests that the acylation conditions in vivo are more selective. Perhaps, the concentration of decanoyl-ACP is much lower than that of lauroyl-ACP in cells.

### Substrate Specificity of the Partially Purified Lauroyl Transferase—Lipid A isolated from E. coli K12 is primarily hexa-acylated (Fig. 1), but a minor penta-acylated species that lacks myristate is often observed (8). This finding suggests that, at least in vivo, the addition of laurate occurs before the addition of myristate. In crude extracts, lauroyl-ACP is a more efficient acyl donor than myristoyl ACP (see above). To determine the acyl chain specificity of the purified lauroyl transferase, acyl-ACPs were not very effective substrates (Fig. 7). The fact that decanoate is not present in lipid A isolated from living cells (8) suggests that the acylation conditions in vivo are more selective. Perhaps, the concentration of decanoyl-ACP is much lower than that of lauroyl-ACP in cells.

### Table III

| Sample          | Total Volume (ml) | Total Protein (mg) | Specific Activity (nmol/min/mg) | Yield (%) |
|-----------------|-------------------|--------------------|-------------------------------|-----------|
| Membranes       | 3.8               | 69                 | 263                           | 100       |
| Thesit extract  | 7.4               | 20                 | 754                           | 84        |
| Thesit insoluble| 5.2               | 49                 | 11.7                          | 2         |
| DEAE-Sepharose  | 1.8               | 3.1                | 2220                          | 37        |

### Figure 4

**Fractionation of the solubilized lauroyl transferase on DEAE-Sepharose CL-6B.** The solubilized membrane proteins (20 mg) were applied onto a 45-ml column as described in the text in detail. Proteins were eluted with a linear NaCl gradient from 50 to 500 mM NaCl. Absorbance at 280 nm (open circles) was used as a measure of total protein in each fraction. Lauryl transferase activity (solid circles) was assayed with Method I using 5 µl of a 1:10 dilution of each fraction. Dotted line, calculated NaCl concentration. Gray area, active fractions that were pooled and concentrated.

### Figure 5

**Substrate Specificity of the Partially Purified Lauroyl Transferase.** A diagram showing the specificity of the partially purified lauroyl transferase for different acyl chain lengths. The enzyme is highly specific for long-chain fatty acids, with laurate being the most effective donor. The addition of shorter-chain fatty acids results in lower activity.

### Figure 6

**Substrate Specificity of the Partially Purified Lauroyl Transferase.** A diagram showing the specificity of the partially purified lauroyl transferase for different acyl chain lengths. The enzyme is highly specific for long-chain fatty acids, with laurate being the most effective donor. The addition of shorter-chain fatty acids results in lower activity.
but this is catalyzed by the msbB gene product (22).

Additional Characterization of Acylated Product a—In previous work with crude extracts, we demonstrated that product a had the mass expected for (Kdo)2-lipid IVA acylated with a single lauroyl group (10). The site of laurate attachment is expected to be the same as in lipid A isolated from cells (7, 8), but this point remains to be established. Treatment of product a with aqueous triethylamine at 37°C results in the release of two R-3-hydroxymyristoyl chains, as judged by thin layer chromatography (data not shown). This behavior is consistent with the attachment of the lauroyl residue to one of the N-linked R-3-hydroxymyristoyl groups, since attachment to one of the O-linked R-3-hydroxymyristoyl groups would prevent its release by aqueous triethylamine (13, 36, 37).

DISCUSSION

Although previous studies of htrB have strongly suggested that this gene has a role in envelope assembly (17–21), the current findings offer the first direct evidence that HtrB is an enzyme of lipid A biosynthesis. Given the pleiotropic nature of the htrB mutant phenotype, Karow and co-workers considered several possibilities for HtrB function, including roles in peptidoglycan (17), fatty acid (20), and lipopolysaccharide biogenesis (19–21). Although they did not propose a primary defect in the Kdo-dependent lauroyl transferase documented in the present work, several earlier observations support our enzymatic data. Specifically, the striking reduction in the laurate content

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of the lipopolysaccharide isolated from htrB deficient E. coli (20) and the reduced number of acyl chains attached to the lipid A of htrB deficient H. influenzae (38) are in accordance with our findings.

MsbB and htrB display significant sequence similarity to each other (19), consistent with related catalytic roles (22). MsbB and htrB have no homology to LpxA (39, 40) and LpxD (31), the products of which are the R-3-hydroxymyristoyltransferases that function in the initial stages of lipid A assembly (3, 5). All four enzymes are absolutely dependent upon acyl chain activation by ACP, since coenzyme A thioesters do not serve as substrates (10, 31, 39). Consequently, they may share some common structural motifs. This issue is currently of considerable interest, since the x-ray crystal structure of LpxA (the first enzyme of the lipid A pathway) has recently been solved at 2.6 Å (41). A unique feature of LpxA is its unusual left-handed parallel β helix domain and trimeric subunit structure (41). HtrB and MsbB do not possess the sequence repeat associated with the left-handed parallel β helix region (41–43), but this does not necessarily rule out the possibility of a β helical domain. Imperfect, parallel right-handed β helices (with no obvious associated sequence repeats) have been reported (44).

HtrB and MsbB, unlike LpxA (45) and LpxD (31), are membrane proteins. X-ray crystallography may not be feasible. In previous studies, however, we reported that half of the lauroyl transferase activity was in the cytoplasmic fraction (10). Different E. coli strains and buffers were employed. Solubilization and purification of HtrB without detergents deserves further exploration.

Both htrB and msbB are present in the Haemophilus genome and show ~70% identity with their E. coli counterparts (46), HtrB and msbB display no sequence homology to other well-characterized acyltransferases, such as glycerol-3-phosphate acyltransferases (46) or N-myristoyltransferases (47). The eucaryotic acylxoyacyl hydrolase (48) that removes the acyl chains incorporated into lipid A by HtrB and MsbB is also unrelated, as judged by its sequence.

The significance of the phenotypes associated with insertions in htrB (17, 19) and the possible functions of multi-copy suppressors of htrB mutations (19, 21) must be re-evaluated in light of our findings. The increased resistance of these mutants to deoxycholate and the stimulation of their growth at non-permissive temperatures by low levels of cationic detergents (19) may be attributed to changes in lipid A structure. The morphological changes at elevated temperatures (17, 19), such as the bulging of the cell surface that resembles the response to certain β-lactams, are more difficult to explain. One intriguing scenario is that underacylated lipid A precursors might accumulate in the inner membrane and inhibit the export of peptidoglycan precursors, preventing peptidoglycan assembly. The msbA multi-copy suppressor of htrB (a putative transporter resembling mammalian mdr proteins) (21) might be the proposed lipid A flipase of the inner membrane (2, 5, 49, 50). The notion of a lipid A translocation function for MsbA is supported by the recent discovery that the mdr-2 protein of the mouse is required for phosphatidylinositol secretion into bile (51). It is conceivable that the overexpression of msbA in htrB-deficient mutants might remove excess, underacylated lipid A precursors by accelerating their export to the outer membrane.

Alternatively, MsbA could be an inner membrane translocase specific for peptidoglycan precursors (52). Its overexpression in the setting of htrB mutations might overcome any inhibitory effects of underacylated lipid A precursors on peptidoglycan export. Further characterization of MsbA function is of great interest, since neither the lipid A nor the peptidoglycan translocases have been identified by biochemical or genetic criteria.

The function of lipid A in Gram-negative bacteria is unknown (1, 2, 5). Strains with insertion mutations in the genes encoding the early steps of lipid A assembly cannot be grown under any condition (53, 54). Wild-type E. coli lipid A contains two normal fatty acids in acylxoyacyl linkage (Fig. 1). Lipid A of some other Gram-negative bacteria possess only one acylxoyacyl unit (1, 2, 4). An important implication of the present studies is that the usual acylxoyacyl moieties of E. coli lipid A are not absolutely essential for growth. Strains with htrB insertion mutations can grow slowly on nutrient media below 33 °C or at all temperatures on minimal media (17, 19). Cells with insertion mutations in msbB show myristate-deficient lipid A and are not conditionally lethal (20, 22, 55). Such
mutants still possess the laurate-containing acyloxyacyl group generated by htrB. To evaluate the significance of acyloxyacyl moieties more thoroughly, a detailed analysis of the molecular species of lipid A that remain in growing htrB mutants of E. coli and in the presence of various suppressors will be of considerable interest. The msbB high multi-copy suppressor of htrB (19) may be functioning by allowing the synthesis of the myristate-containing acyloxyacyl unit that is not made in wild-type cells prior to HtrB catalyzed laurate incorporation (Fig. 1). In addition, palmitate-containing acyloxyacyl moieties can be generated by transacylation of palmitate from the 1 position of a glycerophospholipid to lipid A precursors (28). The latter pathway is of minimal significance in wild-type cells. It is stimulated under conditions of limited lipid A biosynthesis (5, 13, 56). It is possible that a deficiency in laurate incorporation due to an htrB mutation is compensated for by increased palmitate transfer. Further biochemical characterization of htrB mutations and their various suppressors should greatly enhance our understanding of lipid A biosynthesis and function.

Acknowledgment—We thank Dr. Margaret Karow for providing the htrB and msbB related strains listed in Table I and for many helpful discussions.

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