Regulation of UDP-3-O-[R-3-hydroxy-myristoyl]-N-acetylglucosamine Deacetylase in *Escherichia coli*

THE SECOND ENZYMATIC STEP OF LIPID A BIOSYNTHESIS*

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The first enzyme of lipid A assembly in *Escherichia coli* is an acyltransferase that attaches an R-3-hydroxymyristoyl moiety to UDP-GlcNAc at the GlcNAc 3-OH. This reaction is reversible and thermodynamically unfavorable. The subsequent deacetylation of the product, UDP-3-O-[R-3-hydroxy-myristoyl]-GlcNAc, is therefore the first committed step of lipid A biosynthesis. We now demonstrate that inhibition of either the acyltransferase or the deacetylase in living cells results in a 5–10 fold increase in the specific activity of the deacetylase in extracts prepared from such cells. Five other enzymes of the lipid A pathway are not affected. The elevated specific activity of deacetylase observed in extracts of lipid A-depleted cells is not accompanied by a significant change in the $K_M$ for the substrate, but is mainly an effect on $V_{\text{max}}$. Western blots demonstrate that more deacetylase protein is indeed made. However, deacetylase messenger RNA levels are not significantly altered. Inhibition of lipid A biosynthesis must either stimulate the translation of available mRNA or slow the turnover of pre-existing deacetylase. In contrast, inhibition of 3-deoxy-D-manno-octulosonic acid (Kdo) biosynthesis has no effect on deacetylase specific activity. The under-acylated lipid A-like disaccharide precursors that accumulate during inhibition of Kdo formation may be sufficient to exert normal feedback control.

The biosynthesis of lipid A in *Escherichia coli* and related Gram-negative bacteria is catalyzed by nine enzymes (1–3). The identification and characterization of these enzymes followed our discovery of acylated monosaccharide precursors of lipid A, which accumulate in certain phosphatidylglycerol-depleted mutants of *E. coli* (4–7). Despite progress with the biosynthesis, the mechanisms involved in the export of lipid A and the regulation of its assembly remain unknown (1, 3, 8).

In previous studies of temperature-sensitive mutants defective in *lpxA* gene product (10, 11), we found that the specific activity of the second enzyme of the pathway, a deacetylase (see Scheme 1) encoded by *lpxC* (*envA*), is elevated 5–10 fold in extracts of such mutants (12). This finding suggests that the mutant cells may be compensating for the ~30% reduction of the lipid A content that is associated with the *lpxA2* mutation under permissive conditions (11). Regulation of the lipid A pathway at the deacetylase step is reasonable, given that the acylation of UDP-GlcNAc is reversible (12) and that deacetylation represents the first committed reaction (see Scheme 1). A high specific activity of deacetylase is also encountered in extracts of temperature-sensitive mutants defective in the third enzyme of lipid A assembly (see Scheme 1), the *lpxD* (*firA*) gene product (12, 13).

Enzymes catalyzing committed reactions of biosynthetic pathways are often subject to regulation. One of the best characterized examples in membrane lipid synthesis is that of 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A reductase, a key step in sterol formation. HMG-coenzyme A reductase of animal cells is regulated by proteolysis (14–17), by phosphorylation (18), and at the level of transcription (14, 19–21). How cells sense their sterol content is still not entirely clear. However, the transcription factors that are involved in sterol-dependent regulation in animal cells have been identified (19–21). In yeast, there are two HMG-coenzyme A reductase isoenzymes that are regulated by different transcriptional and post-transcriptional mechanisms (22).

We now demonstrate that increased amounts of UDP-3-O-[R-3-hydroxy-myristoyl]-GlcNAc deacetylase (see Scheme 1) are made in *E. coli* under conditions that reduce the lipid A content of cells. Elevated deacetylase levels are observed not only in conditional mutants (11, 12) defective in UDP-GlcNAc acyltransferase (lpxA), but also in wild-type cells treated with a specific inhibitor of the deacetylase. Western blots indicate that more enzyme protein is present in both cases. The observed effects are not the result of increased transcription of the gene (*lpxC*) (24) coding for the deacetylase. Our results show that *E. coli* cells possess novel mechanisms for sensing lipid A-like molecules in their envelopes and for regulating the rate of lipid A biosynthesis accordingly.

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downstream of the first committed reaction. The known genes encoding the enzymes of the pathway are indicated (2, 3). The structures of the intermediates have all been elucidated and have been reviewed elsewhere (2, 3, 54). The first step, catalyzed by UDP-GlcNAc deacetylase, is the second enzyme of the lipid A pathway, but because the deacetylase is actually thermodynamically unfavorable (12), the deacetylase is actually the second committed reaction. As described in the legend to Fig. 1. The deacetylase-driven overproducer of acyl-ACP synthetase, was obtained from Dr. A. Patchett (Merck Research Laboratories, Rahway, NJ), and the CMP-Kdo synthase inhibitor (25, 26) was obtained from Dr. A. Patchett (Merck Research Laboratories, Rahway, NJ). Silica Gel 60 thin layer plates (0.25 mm) were purchased from Merck (Darmstadt, Germany). PhosphorImager screens were from Molecular Dynamics, Inc. Formamid, salmon sperm DNA, and RNA standards were obtained from Life Technologies, Inc. All other chemicals were purchased from Sigma. The deacetylase inhibitor L-573,655 was obtained from Dr. A. Patchett (Merck Research Laboratories, Rahway, NJ), and the CMP-Kdo synthase inhibitor (25, 26) was obtained from Dr. R. Goldman (Abbott Laboratories, North Chicago, IL).

Bacterial Strains—All strains used in this study were derived from E. coli K12, SM101, SM105, and SM108 have been described previously (11, 27). W3110 was obtained from the E. coli Genetic Stock Center, Yale University (New Haven, CT). LCH109/pLCH5/pGP1-2, a T7 promoter-driven overproducer of acyl-ACP synthetase, was obtained from Dr. C. O. Rock (St. Jude’s Hospital) (28). JBB-1/pKD6 was constructed as described in the legend to Fig. 1. The lipA (enA) gene on the chromosome of JBK-1 was disrupted by a transposon, but pKD6 harbors the 2.5-kilobase chromosomal EcoRI fragment containing the complete lipA sequence with its normal promoter (29). pKD6 (8.5–9 kilobases) was derived from pELE3 (Fig. 1), a plasmid with a temperature-sensitive replication that is maintained at low copy number at 30 °C (30). Since lipA is an essential gene, JBK-1/pKD6 is temperature-sensitive for growth.

Preparation of Cell Extracts—When not otherwise stated, cell-free extracts for activity measurements were prepared as follows. A single colony was inoculated into 5 ml of Luria broth (31) and grown overnight at 30 or 37 °C. A larger culture of Luria broth (100–500 ml) was inoculated by 100-fold dilution of the overnight culture and grown at 30 or 37 °C to late log phase (A600 = 1.0). The cells were harvested by centrifugation at 7000 × g for 10 min at 2 °C, washed once with 0.1 volume of cold 10 mM Heps (pH 7.5), and resuspended in a minimal volume (usually 2–3 ml) of the same buffer. An ice-cold French pressure cell at 18,000 p.s.i. was used to disrupt the cells. Unbroken cells were removed by centrifugation at 7000 × g for 10 min at 2 °C. Aliquots of the supernatant were stored at −80 °C.

Preparation of R-3-Hydroxymyristoyl-ACP—The substrate for R-3-hydroxymyristoyl-ACP was prepared from purified ACP (Sigma) and synthetic R-3-hydroxymyristoyl-ACP (22), using Triton X-100-solubilized LCH109/pLCH5/pGP1-2 membranes as the source of acyl-ACP synthetase (28, 33).

The enzymatic acylation of ACP with R-3-hydroxymyristoyl-ACP was performed in 600-μl microcentrifuge tubes at 30 °C in a final volume of 20 μl. The reaction mixture contained the following components: 3 mM [α-32P]UDP-GlcNAc and [α-32P]UDP-3-O-[R-3-hydroxymyristoyl]-GlcNAc (1 μl each) prepared as described previously with minor modifications (3). In the [α-32P]UDP-GlcNAc preparation, the triethylammonium salt was exchanged by dialysis into distilled H2O using a Centricon-3 membrane (Amicon, Inc.). The acyl-ACP was ~90% pure, as judged by electrophoresis in the urea-polyacrylamide gel system (34) and staining with Coomassie Blue.

Synthesis of [α-32P]UDP-GlcNAc and [α-32P]UDP-3-O-[R-3-hydroxymyristoyl]-GlcNAc—[α-32P]UDP-GlcNAc and [α-32P]UDP-3-O-acyl-GlcNAc were prepared as described previously with minor modifications (15). In the [α-32P]UDP-GlcNAc preparation, the triethylammonium salt was exchanged by dialysis into distilled H2O using a Centricon-3 membrane (Amicon, Inc.). [α-32P]UDP-GlcNAc was prepared as described above. Assay mixtures (20 μl) contained 200 μM [α-32P]UDP-GlcNAc (1 × 106 cpm/nmol), 50 μM R-3-hydroxymyristoyl-ACP, 1% acetyl β-D-glucoside (to inhibit the deacylase in crude extracts), 40 mM Hepes (pH 8.0), and 1.0 mg/ml cell extract. The reaction was initiated by adding extract and placing the tubes at 30 °C. A 5-μl sample was removed from the reaction and mixed with 1 μl of 12.5% NaOH in a second microcentrifuge tube to stop the reaction. The tubes were then incubated at 30 °C for 10 min to remove ester-linked fatty acids. These samples were neutralized with 1 μl of 1.25 M acetic acid and 1 μl of 5% trichloroacetic acid. The tubes were placed on ice for 5 min and then centrifuged for 2 min. A 2-μl portion of supernatant was spotted onto a flexible polyethyleneimine-cellulose plate. The plate was washed, dried in a Speed Vac centrifuge rather than being lyophilized. UDP-3-O-acetyl-GlcNAc Deacylase Assay—The deacylase assays were performed in 600-μl microcentrifuge tubes at 30 °C in a final volume of 20 μl. The reaction mixture contained the following components: 3 mM [α-32P]UDP-3-O-[R-3-hydroxymyristoyl]-GlcNAc (2.6 × 1010 cpm/nmol), 40 mM Tris-HCl (pH 8.0), and 1.0 mg/ml cell extract. The reaction was initiated by adding the cell extract and immediately placing the tubes at 30 °C. After 4 and 8 min, a 5-μl sample was removed from the reaction and mixed with 1 μl of 12.5% NaOH in a second microcentrifuge tube to stop the reaction. The tubes were then incubated at 30 °C for 10 min to remove ester-linked fatty acids. These samples were neutralized with 1 μl of 1.25 M acetic acid and 1 μl of 5% trichloroacetic acid. The tubes were placed on ice for 5 min and then centrifuged for 2 min. A 2-μl portion of supernatant was spotted onto a flexible polyethyleneimine-cellulose plate. The plate was washed, dried in a Speed Vac centrifuge rather than being lyophilized. UDP-3-O-acetyl-GlcNAc was prepared as described above. Assay mixtures (20 μl) contained 200 μM [α-32P]UDP-GlcNAc (1 × 106 cpm/nmol), 50 μM R-3-hydroxymyristoyl-ACP, 1% acetyl β-D-glucoside (to inhibit the deacylase in crude extracts), 40 mM Hepes (pH 8.0), and 1.0 mg/ml cell extract. The reaction was initiated by adding extract and placing the tubes at 30 °C. A 5-μl portion of the reaction mixture was spotted after 2 and 5 min onto a silica thin layer plate. The plate was developed with chloroform/methanol/water/acetie acid (25:15:4:2, v/v), and the extent of acylation was determined using a PhosphorImager.

Disaccharide Synthase Assay—[32P]-Labeled lipid X (35) was used as the substrate for this assay, and cell extracts were assayed as described.
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Fig. 1. Map of plasmid pKD6 and construction of E. coli strain JBR-1/pKD6. pKD6 was constructed by cloning a 2.5-kilobase (Kb) EcoRI fragment containing the lpxC (envA) gene from pBLS (29) into the temperature-sensitive replicon (Rep (Ts)) pEL3 (30), in which an EcoRI site had been introduced at the single Kan allele from BL7623 (kan) site. pKD6 was selected by complementation of lpxC (envA) mutant strain GL5003 at 30°C (29). The strain JBR-1/pKD6 contains the chromosomal lpxC gene disrupted with a kan element and a wild-type copy of lpxC on the temperature-sensitive replicon pKD6. This strain was constructed by transducing the lpxC::kan allele from BL7623 (ΔlpxC-29) into W3110/pKD6, selecting for kan resistance at 30°C, and screening for temperature sensitivity. Subsequently, the recA56 mutation was introduced by cotransduction with srl::Tn10.

Earlier (35), the total reaction volume was 10 μl. Reaction mixtures contained 10 mM Hepes (pH 8.0), 0.5 mM 32P-labeled lipid X (1 × 106 cpm/nmol), 0.5 mM UDP-2,3-diacyl-GlcN, 0.2 mM fatty acid-free bovine serum albumin, and 1.0 mg/ml crude cell extract. The reaction was stopped by spotting 5 μl of reaction mixture onto a silica thin layer plate after 15 and 30 min. The plate was developed with chloroform/methanol/water/acetic acid (25:15:4:2, v/v), and disaccharide formation was estimated from the position of the radioactive band on the plate. The plate was developed with chloroform/methanol/water/acetic acid (25:15:4:2, v/v), and disaccharide formation was estimated from the position of the radioactive band on the plate.

Late Acylation Assay—The substrate for this reaction, 1-32P-labeled disaccharide monophosphate, was synthesized as described previously (36). Cell extracts were assayed by a method similar to that of Hampton and Raetz (36), with minor modifications. The assay mixtures (20 μl) contained 0.25 mg/ml disaccharide monophosphate (6.8 × 105 cpm/assay tube), 100 mM Tris-HCl (pH 8.5), 2 mg/ml cardiolipin, 1% Triton X-100, 5 mM MgCl2, 10 mM ATP, and 1.0 mg/ml crude cell extract. Samples were removed at 5 and 15 min and analyzed for product formation by thin layer chromatography (36). The extent of 4'-phosphorylation was determined using a PhosphorImager.

Kdo Transferase Assay—4'-32P-Labeled lipid IVX, which was used as the substrate for this assay, was isolated and purified as described earlier (36–38). The cell extracts were assayed essentially as described by Brozek et al. (39). The total reaction volume was 20 μl. 4'-32P-Labeled lipid IVX was used at 100 μM (3 × 105 cpm/nmol) in 50 mM Hepes (pH 7.0). Crude cell extract was used as the source of enzyme at 0.2 mg/ml. Samples were removed at 10, 20, and 30 min, and Kdo transfer was determined after thin layer chromatography (39, 40) using a PhosphorImager.

Western Blots—The cells used for the Western blots were grown until they reached late log phase (A600 = 1.0). In one set of experiments (as indicated in the figure legends), cells from 1 ml of culture were collected using a microcentrifuge and resuspended in 300 μl of 2-fold concentrated Laemmli sample buffer (42). The samples were boiled for 90 s and immediately placed on ice. They were then centrifuged for 20 min at 14,000 rpm, and the pellet was removed. Next, 25-μl portions of the supernatants (~12 μg of protein) were analyzed on a 10% SDS-polyacrylamide gel (42) at 50 mA until the bromphenol blue reached the bottom of the gel. Bio-Rad protein molecular weight standards low range molecular weight standards and 200 ng of purified deacetylase protein were also analyzed on the same gel. The gel was equilibrated in 10 mM CAPS (pH 11.0) at 4°C, and then the proteins were transferred to a nitrocellulose membrane using a Transblot Semi-Dry Transfer Cell (Bio-Rad) at 20 V for 40 min. The membrane was incubated in ~40 ml of blocking buffer (phosphate-buffered saline (pH 7.5), 1% Koger nonfat dry milk, and 0.2% Tween 20) for 1 h with gentle shaking. Primary antibody was added to the blocking buffer by ~1: 5000-fold dilution from a stock, and the incubation was continued for an additional hour. The primary antibody used in the Western blots consisted of rabbit serum (~40 mg/ml) that had been filtered through a total E. coli protein column (Pierce). The membrane was then rinsed for 1 h with four buffer changes of washing buffer (phosphate-buffered saline (pH 7.5) and 0.2% Tween 20). Secondary antibody was diluted 5000-fold from a 0.5 mg/ml stock into the blocking buffer. This solution (~40 ml) was added to the membrane and incubated for 1 h. The membrane was washed in the same way as described above and developed using enhanced chemiluminescence reagents.

In other experiments, cell-free extracts were prepared first by passage through a French press cellul. Portions of these extracts were then mixed with Laemmli sample buffer (42) and analyzed as described above. The primary antibody used was a polyclonal rabbit antibody generated from purified deacetylase protein (24). The antiserum was produced at HazeloN Research Products Inc. (Denver, PA). The secondary antibody was donkey antirabbit immunglobulin conjugated with horseradish peroxidase (Amersham Corp.).

Northern Blot—The Northern blot was done as described (43). The 32P-labeled DNA probe for the deacetylase mRNA was constructed by first using PCR to amplify the lpxC gene off of the pKD6 plasmid. Briefly, 1 ng of pKD6 DNA and two custom primers were used with a GeneAmp kit (Perkin-Elmer) to set up the PCR. The sequences of the primers used were as follows: 5'-PACCGATATCAACAAAGGGACAC-3' and 5'-PCTATGCGCATACGCATGAAGGCCG-3'. The PCR went through 35 cycles: 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. Then, 110 μl of the PCR was analyzed on a 1% agarose gel that was run at 100 V for ~3 h. The PCR product was purified using Geneclean (BIO 101, Inc.) to yield 40 μg of DNA (25 ng of this DNA was used as the template for the Prime-I H random primer labeling kit (Stratagene). The labeling reaction was done with [α-32P]dATP. The probe was purified away from contaminating free nucleotides by using a NucTrap probe purification column (Stratagene). Unfractionated RNA was isolated from E. coli by using an RNasy Total RNA isolation kit (QIAGEN Inc.). Cells grown to A600 = 1.0 (1 ml culture in 10° cells was used at 0.2% Tween 20 for 1 h with a 32P-label was added, and A600 of the samples was used to calculate the amount of RNA recovered, and A600/A600 was determined to estimate the quality of the RNA. The A600/A600 ratio for all of the samples was between 1.9 and 2.1, and 10 μg of each RNA sample was used for the Northern blot. RNA standards were used to estimate the size of the transcript. The Northern blot was analyzed using a PhosphorImager.

Construction of a β-Galactosidase-based Plasmid for Assaying lpxC Promoter Activity—The 146-base pair region immediately in front of the lpxC gene (29) was amplified by polymerase chain reaction and placed in the β-galactosidase-based promoter detection vector pRL124 (ATCC 37683). Primers for amplification contained GC clamps and restriction sites allowing unidirectional cloning into the pUCI and EcoRI sites of pRL124 in the sense (5′-GGCG CGTC ACG ATA TCC CAG CAT-3′) and antisense (5′-CGG GAA ATG GTT ACC TGC CC-3′) directions, respectively. This PCR fragment was generated using genomic DNA of E. coli strain R477 (44) as template. Conditions for PCR were as follows: taq polymerase (Perkin-Elmer) in a thermocycle series of 96°C for 30 s, 40°C for 30 s, and 72°C for 2 min for 35 cycles, followed by a final 10 min at 72°C. The fragment generated was cut with an excess of restriction enzymes for 2 h at 37°C, purified from a 1.3% agarose gel (FMC Corp., BioProducts), and recovered using Qiaex resin (QIAGEN Inc.). This fragment was ligated into similarly cut pRL124 and transformed into XL-1 Blue (Stratagene). One isolate containing a properly sized fragment as judged by restriction digestion was chosen and designated pRS146.

This plasmid isolate was also transformed into SM101 and SM105 in parallel by electroporation (Bio-Rad), and a colony of each was repurified under ampicillin selection. Extracts were prepared from transformants grown in LB medium as described above, with the exception that harvested cells were not washed in order to minimize any loss of β-galactosidase activity. Rather, cells were directly resuspended in a
buffer consisting of 10 mM sodium phosphate (pH 7.0) and lysed by a single passage through a French pressure cell at 18,000 p.s.i. Debris was removed by centrifugation at 15,000 × g for 20 min. β-Galactosidase activity was measured in a microtiter plate, using several dilutions of the extract in the assay mixture of Miller (31). Assay mixtures were incubated at 37 °C for 30 min, and the resulting absorbance was read at 405 nm. Activity was calculated by comparison with a o-nitrophenol standard curve.

**RESULTS**

Elevation of UDP-3-O-[R-3-hydroxyxymiristoyl]-N-acetylgalactosamine Deacetylase Activity in Mutant SM101—In previous studies, we have shown that SM101 harbors a point mutation in the lpxA gene in which Gly-189 is replaced by Ser (11, 27). The lpxA gene encodes UDP-GlcNac 3-O-acyltransferase, the first enzyme of the lipid A pathway (Scheme 1). The mutant allele (lpxA2) renders growth and lipid A biosynthesis temperature-sensitive in SM101 (11, 27). However, even at the permissive temperature (30 °C), SM101 displays a 30% reduction of its lipid A content (11, 27), and SM101 is hypersensitive to antibiotics, like rifampicin, that normally are excluded by the outer membrane (45, 46).

As shown in Table I, extracts of SM101 grown at 30 °C are characterized by a 8-fold increase in the specific activity of UDP-3-O-[R-3-hydroxyxymiristoyl]-N-acetylgalactosamine deacetylase, the second enzyme of the lipid A pathway (Scheme 1). In extracts of cells held for several hours at 42 °C, the specific activity of the deacetylase is elevated 12-fold (data not shown). Mixing of equal amounts of wild-type (SM105) and mutant (SM101) extracts results in additive specific activities shown). Mixing of equal amounts of wild-type (SM105) and specific activity of the deacetylase is elevated 12-fold (data not shown). In extracts of cells held for several hours at 42 °C, the specific activity of the deacetylase is elevated 12-fold (data not shown).

**TABLE I**

| Enzymes                              | SM105a (lpxA2) | SM105b (lpxA2) | SM108a (lpxA2) | SM108b (lpxA2) |
|--------------------------------------|----------------|----------------|----------------|----------------|
| UDP-GlcNac O-acyltransferase         | 1.50           | 0.03           | 0.07           |
| Deacetylase                          | 0.19           | 1.48           | 0.05           |
| Disaccharide synthase                | 3.50           | 3.17           | 3.85           |
| 4'-Kinase                            | 0.80           | 0.91           | 1.06           |
| Kdo transferase                      | 1.35           | 1.60           | 1.20           |
| Lauroyltransferase                   | 2.07           | 2.65           | 1.52           |

a All extracts were assayed at 30 °C.

b Cells were grown at 30 °C to late log phase.

Increased Specific Activity of Deacetylase in Extracts of Wild-type Cells Treated with L-573,655—The R-isomer of the compound L-573,655 is a competitive inhibitor of the UDP-3-O-[R-3-hydroxyxymiristoyl]-N-acetylgalactosamine deacetylase of *E. coli* and other Gram-negative bacteria. Like the lpxA2 mutation, L-573,655 selectively inhibits the formation of lipid A in living cells. As shown in Fig. 2, a dose-dependent increase in the specific activity of the deacetylase is observed in extracts of cells exposed to L-573,655 for several hours. As in the case of the lpxA2 mutation (Table I), the maximal increase in deacetylase activity is ~10-fold (Fig. 2). When the inhibitor concentration is increased above 16 μg/ml, the specific activity of the deacetylase no longer increases, and inhibition of cell growth sets in. Since accumulation of deacetylase activity is seen in extracts of wild-type cells exposed to L-573,655 (Fig. 2), the effect of inhibition of lipid A biosynthesis on the specific activity of the UDP-GlcNac O-acyltransferase (the lpxA gene product) can also be examined. As shown in Fig. 2, the specific activity of UDP-GlcNac O-acyltransferase is not affected by L-573,655, indicating that the cellular response to lipid A inhibition is restricted to the deacetylase (Fig. 2 and Table I). Other Gram-negative bacteria, including strains of *Enterobacter cloacaes*, *Proteus mirabilis*, *Serratia marcescens*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, also display increased levels of deacetylase when exposed to L-573,655 (data not shown). Since the *K*<sub>v</sub> of L-573,655 is ~25 μM, it is washed away during the harvesting of the cells and the preparation of the extracts. It therefore does not interfere with the deacetylase assays.

Deacetylase Activation Is Predominantly on Effect on *V*<sub>max</sub>—The radiochemical assay for the deacetylase is sufficiently sensitive to permit the determination of the *K*<sub>m</sub> (μM) and *V*<sub>max</sub> (expressed as nmol/min/mg) in crude cell extracts. As shown in Fig. 3, the high specific activity of the deacetylase in extracts of SM101 is accounted for almost entirely by an effect on *V*<sub>max</sub>, as is the reduced activity in SM108. The observed *K*<sub>m</sub> values (see...
Levels of the precursor, lipid IVA, cause an arrest of cell growth and interrupt the lipid A pathway (25, 26). These compounds deacetylase in cell extracts is not elevated in cells exposed to a.

Inhibition of Kdo Biosynthesis Does Not Increase Deacetylase Levels—As shown in Table II, the specific activity of the deacetylase in cell extracts is not elevated in cells exposed to a CMP-Kdo biosynthesis inhibitor (L-573,655) (25, 26). The compounds cause an arrest of cell growth and interrupt the lipid A pathway because Kdo transfer (Scheme 1) cannot occur.抑制 of CMP-Kdo formation results in the accumulation of high levels of the precursor, lipid IV-A, in vivo (25, 26, 47).

As expected from the activity measurements (Fig. 2), inhibition of the deacetylase in wild-type cells with L-573,655 results in higher levels of deacetylase protein detected by Western blotting (Fig. 5, lane 2). The CMP-Kdo biosynthesis inhibitor does not elevate the amount of deacetylase protein present in wild-type cells (Fig. 5, lane 3).

Disappearance of the Deacetylase at 42 °C in Strain JBK-1/pKD6—Strain JBK-1/pKD6 contains an insertion mutation in the chromosomal copy of the lpxC gene that encodes the deacetylase (see the legend to Fig. 1). The hybrid plasmid pKD6 (Fig. 1) harbors a wild-type lpxC gene, but pKD6 cannot replicate at 42 °C (30). Accordingly, JBK-1/pKD6 is temperature-sensitive for growth. About 4 h after a shift to 42 °C, the cell density stops increasing, and the culture gradually undergoes lysis (data not shown).

As shown in Fig. 6, the specific activity of the deacetylase gradually declines in extracts of JBK-1/pKD6 cells shifted to 42 °C. Prior to the temperature shift (time 0), deacetylase specific activity is 2–3-fold higher than in the wild type (Table I), consistent with the copy number of the hybrid plasmid employed. Loss of deacetylase protein, as judged by Western blotting, accompanies loss of deacetylase specific activity in this setting (Fig. 7), as expected if the enzyme is being diluted out at
Deacetylase activity in extracts of strain JBK-1/pKD6 after a shift to 42°C. The cells used to make extracts for assays of deacetylase specific activity were grown as follows. Four 100-ml cultures (LB medium + 150 μg/ml ampicillin) were inoculated 1:100 from an overnight culture on the same medium, and they were grown at 30°C until A_600 = 0.07. At this point, three cultures were shifted to 42°C. One culture was grown at 30°C and harvested when A_600 reached 1.0. The 42°C cultures were back-diluted 10-fold whenever A_600 reached 0.8 to keep the cells in log phase. The cumulative increase in cell mass was ~100-fold at 42°C before growth stopped (~4 h after the temperature shift). One of the 42°C cultures was harvested at each of the times indicated. Cell extracts were prepared and assayed for deacetylase activity as described under "Experimental Procedures." JBK-1/pKD6 extracts from cells grown at 30°C (time 0) and shifted to 42°C for 2 h were assayed at 0.2 mg/ml cell extract. The two cultures that were held at 42°C for 4 and 5.5 h were assayed at 1.0 mg/ml cell extract.

42°C in the absence of the covering plasmid. The observation that the ~34,000-kDa protein appears in the Western blot after several hours of incubation at 42°C in JBK-1/pKD6 cells (Fig. 7) supports the identification of this band as the deacetylase protein in the experiments of Figs. 4 and 5.

Deacetylase mRNA Levels Are Not Significantly Elevated in SM101—A Northern blot of 10-μg RNA samples extracted from SM101, SM105, and SM108 cells is shown in Fig. 8 (lanes I–3, respectively). A hybridizing band is observed at the position expected for lpxC mRNA (~1100 nucleotides), as indicated. This mRNA size is predicted from the lpxC DNA sequence (29). Its abundance in SM101 (Fig. 8, lane 1) is about the same as in SM105 (lane 2) and SM108 (lane 3). A duplicate experiment (not shown) confirmed these results.

Fig. 8 (lanes 4–6) also shows that the same 1100-nucleotide band is detected in 10-μg RNA samples extracted from JBK-1/pKD6 cells. However, in cells grown at 30°C (Fig. 8, lane 4), the intensity of the message is much greater than after 2 (lane 5) or 4 (lane 6) h of cell growth at 42°C. The disappearance of this mRNA species in JBK-1/pKD6 cells at 42°C is consistent with the activity data (Fig. 6) and supports the identification of this band as the mRNA encoding the deacetylase.

A β-galactosidase reporter gene fused to the normal lpxC promoter (29) was introduced into SM101 and SM105 on a hybrid plasmid (Table III). If transcription of lpxC is enhanced in SM101, a higher specific activity of β-galactosidase would be observed in the presence of the lpxA2 mutation. As shown in Table III, there is no enhanced transcription of this reporter gene in SM101 compared with SM105. These findings are consistent with the Northern blot analysis of SM101 and SM105 shown in Fig. 8. Treatment of wild-type cells with L-573,655 also failed to elevate the levels of deacetylase mRNA, as judged by Northern blotting (data not shown) and reporter gene analysis (Table III).

DISCUSSION

Previous studies of the regulation of membrane lipid composition in E. coli have focused on three phenomena. These are as follows: 1) the regulation of fatty acid degradation and synthesis at the level of transcription by the fabR repressor (48–50), 2) the regulation of fatty acid composition as a function of growth temperature mediated by the fabF gene (50, 51), and 3) the stimulation of glycerophospholipid turnover and membrane-derived oligosaccharide synthesis at low osmolarity (52). The discovery that deacetylase levels are controlled over a 20-fold range in relation to the lipid A content appears to be a new regulatory phenomenon. It is independent of temperature, osmolarity, and the fabR gene. A comparable regulatory mechanism has not been described for glycerophospholipids (53–55).

However, trans-acting mutations have been reported that elevate the levels of specific glycerophospholipid synthetic enzymes, such as diacylglycerol kinase (56) and phosphatidylserine synthase (57).

Deacetylase regulation in E. coli may share some common features with HMG-coenzyme A reductase regulation in euca- ryotic cells. In both cases, the amount of enzyme increases when the synthesis of a major surface membrane lipid is blocked. In both cases, either an enzyme inhibitor or a mutation in an earlier step in the pathway (22) can cause enzyme induction. These findings exclude the possibility that the inhibitors are simply stabilizing their respective target enzymes against degradation. A key difference is that deacetylase regulation does not appear to be based on the control of transcription. However, HMG-coenzyme A reductase regulation does include an important non-transcriptional component, involving specific proteolysis of HMG-coenzyme A reductase in response
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sensor(s), one could examine mutants that are hypersensitive to the proposed regulatory system of procaryotes (62, 63). To identify these model A, a protein required for translation is inactivated by lipid A. This results in the production of more deacetylase when lipid A levels are low. In model B, a protease that can degrade the deacetylase is activated by lipid A, accounting for high deacetylase levels when the lipid A content is reduced. In both cases, we propose that the disaccharide bisphosphate precursors of lipid A that accumulate during inhibition of Kdo biosynthesis (47, 58–60) are sufficient to function as regulatory signals since inhibition of Kdo biosynthesis does not induce the deacetylase (Table II). The proteolytic scenario is especially attractive because there is a well-characterized precedent for lipid A-activated proteolysis in the clotting system of the Limulus crab (61). To distinguish between these models, we could determine the rates of enzyme synthesis and turnover under conditions of limited lipid A formation.

The models proposed in Fig. 9 postulate that the putative translation factor or protease itself is the lipid A sensor. A viable alternative is that a distinct lipid A sensor exists in the outer membrane or on the periplasmic surface of the inner membrane. The putative lipid A sensor might transmit a second message that is responsible for the regulation of the deacetylase, in analogy to the functioning of other two-component regulatory systems of procaryotes (62, 63). To identify the genes encoding the putative lipid A-responsive protein(s) or sensor(s), one could examine mutants that are hypersensitive to L-573,655. Such strains might include mutants that are unable to mount the usual deacetylase response when lipid A biosynthesis is inhibited and therefore would be killed at lower concentrations of the inhibitor compared with wild-type cells. If the proposed protease in model B could mutate to be active in the absence of lipid A, cells might become very hypersensitive to L-573,655 or might even display some kind of conditional lethality.

One could also search directly for lipid A-regulated proteases or translation factors. All the reagents, including the cloned gene, purified protein, and antibodies, are now available to study deacetylase synthesis and turnover.

The possible function of the regulation that we have discovered deserves comment. L-573,655 is not a natural product, and mutations in lpxA are not normally present in Gram-negative bacteria. One could therefore question the biological significance of the observed effects. Under laboratory conditions, the lipid A content of E. coli is ~0.12 mol of lipid A/mol of glycerophospholipid (11), and the ratio does not vary greatly from strain to strain.3 We speculate that physiological conditions may yet be found in which it is necessary to activate lipid A biosynthesis. For instance, it is known that chelating agents, like EDTA, remove a significant fraction of the lipopolysaccharide from the cell surface (9, 23). It is conceivable that natural chelating agents exist that might have the same effect. To survive such stresses, cells might have an advantage if they could increase the production of lipid A. It is also conceivable that the observed regulation of the deacetylase normally operates to match the rate of lipid A synthesis with the growth rate.

Another possible reason for deacetylase regulation is the observation that overproduction of the deacetylase is lethal to cells (24, 29). Moderate overproduction of other enzymes of the lipid A pathway does not inhibit cell growth (2, 3, 54). Because of the potential for toxicity, it may be important for cells to control deacetylase levels within a relatively narrow range. The biochemical basis for deacetylase toxicity is unknown. Perhaps, excess deacetylase shunts too large a fraction of nascent fatty acyl chains into lipid A, resulting in depletion of glycerophospholipids and/or UDP-GlcNAc. In either case, growth arrest would result. Expression of the deacetylase under the control of an artificially regulated promoter may provide insights into the function of deacetylase regulation and may reveal the basis for deacetylase toxicity.

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Regulation of UDP-3-O-[R-3-hydroxymyristoyl]-N-acetylglucosamine Deacetylase in Escherichia coli: THE SECOND ENZYMATIC STEP OF LIPID A BIOSYNTHESIS

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