Effect of Cold Shock on Lipid A Biosynthesis in Escherichia coli

INDUCTION AT 12 °C OF AN ACYLTRANSFERASE SPECIFIC FOR PALMITOLEOYL-ACYL CARRIER PROTEIN*

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Palmitoleate is not present in lipid A isolated from Escherichia coli grown at 30 °C or higher, but it comprises ~11% of the fatty acyl chains of lipid A in cells grown at 12 °C. The appearance of palmitoleate at 12 °C is accompanied by a decline in laurate from ~18% to ~5.5%. We now report that wild-type E. coli shifted from 30 °C to 12 °C acquire a novel palmitoleoyl-acyl carrier protein (ACP)-dependent acyltransferase that acts on the key lipid A precursor Kdo2-lipid IV\(_A\). The palmitoleoyl transferase is induced more than 30-fold upon cold shock, as judged by assaying extracts of cells shifted to 12 °C. The induced activity is maximal after 2 h of cold shock, and then gradually declines but does not disappear. Strains harboring an insertion mutation in the lpxL gene, which encodes the enzyme that normally transfers laurate from lauroyl-ACP to Kdo2-lipid IV\(_A\) (Clementz, T., Bednarски, J. J., and Raetz, C. R. H. (1996) J. Biol. Chem. 271, 12095–12102) are not defective in the cold-induced palmitoleoyl transferase. Recently, a gene displaying 54% identity and 73% similarity at the protein level to lpxL was found in the genome of E. coli. This lpxL homologue, designated lpxP, encodes the cold shock-induced palmitoleoyl transferase. Extracts of cells containing lpxP on the multicopy plasmid pSK57 exhibit a 10-fold increase in the specific activity of the cold-induced palmitoleoyl transferase compared with cells lacking the plasmid. The elevated specific activity of the palmitoleoyl transferase under conditions of cold shock is attributed to greatly increased levels of lpxP mRNA. The replacement of laurate with palmitoleate in lipid A, in mutants defective in Kdo biosynthesis (12–15).

Lipopolysaccharide (LPS) is a major component of the outer leaflet of the outer membrane of Escherichia coli and is a defining feature of Gram-negative bacteria (1–6). LPS consists of three domains: 1) lipid A, the hydrophobic membrane anchor (Fig. 1); 2) the core sugar region; and 3) the O-antigen polymer (1–6). Studies of E. coli mutants in LPS biosynthesis have demonstrated that the lipid A domain and the Kdo residues of the proximal core are required for growth (1–3, 7).

In wild-type E. coli grown at 30 °C or above, lipid A contains six fatty acyl chains (Fig. 1) (3, 4, 8). Four R-3-hydroxymyristate residues are attached directly to the glucosamine disaccharide backbone (3, 4, 8). Laurate and myristate are attached to the R-3-hydroxy groups of the R-3-hydroxymyristate residues in the distal unit (Fig. 1) (3, 4, 9). In the later steps of the lipid A biosynthetic pathway (Fig. 1), the key intermediate Kdo2-lipid IV\(_A\) is sequentially acylated with laurate and myristate (3, 4, 9–11). The lauroyl and myristoyl transferases require the presence of the Kdo disaccharide for optimal activity (9–11). The substrate preferences of these enzymes are consistent with the accumulation of lipid IV\(_A\), rather than lipid A, in mutants defective in Kdo biosynthesis (12–15).

As shown by Clementz et al. (10, 11, 16), the lpxL (litrhB) gene, which was initially identified as being required for growth of E. coli above 32 °C (17, 18), encodes the lauroyl transferase (Fig. 1). We have recently purified LpxL to homogeneity and confirmed that lpxL is indeed the structural gene for the lauroyl transferase. The LpxL reaction product, Kdo2-lauroyl-lipid IV\(_A\), is the preferred substrate for the myristoyl transferase (9, 11), which is encoded by the lpxM gene (previously designated msbB) (Fig. 1) (19). The lpxM (msbB) gene displays distant, but nevertheless significant, sequence similarity to lpxL (19). High copy number plasmids bearing lpxM can suppress the temperature-sensitive growth of mutants defective in lpxL (19), presumably because LpxM acylates Kdo2-lipid IV\(_A\) at a slow rate (11).

In 1979, prior to the elucidation of the structure and biosynthesis of lipid A, van Alphen et al. studied the fatty acid composition of lipid A from E. coli grown at 37 °C versus 12 °C (20). At 37 °C, laurate was present at 0.16 μmol/mg of LPS, but it decreased to 0.05 μmol/mg at 12 °C (20). The decrease in laurate at 12 °C was counterbalanced by the appearance of palmitoleate, which was present at 0.10 μmol/mg of LPS in E. coli cells grown at 12 °C (20). Only trace amounts of palmitoleate were found in LPS at 37 °C. A similar effect of cold shock on lipid A composition was reported for Salmonella typhimurium (21). It is unlikely that palmitoleate incorporation is catalyzed by LpxL, since palmitoleoyl-acyl carrier protein (ACP) is not a substrate for LpxL (9, 10).

We now describe the induction of a novel palmitoleoyl transferase in extracts of E. coli cells subjected to cold shock (Fig. 1). This enzyme transfers palmitoleate from palmitoleoyl-ACP to Kdo2-lipid IV\(_A\), which is also the acceptor for the lpxL-encoded lauroyl transferase (Fig. 1). An inducible palmitoleoyl transferase would account for the appearance of palmitoleate in lipid A of cells grown at 12 °C. The palmitoleoyl transferase is not encoded by lpxL, but appears to be the product of another lpxL homologue found in E. coli (22) that is now designated lpxP.

S. M. Carty and C. R. H. Raetz, manuscript in preparation.
The palmitoleoyl transferase is induced within minutes after cold shock and is accompanied by a massive increase in the levels of lipXp mRNA. A cold shock-induced acyltransferase has not been reported previously.

**Experimental Procedures**

**Materials**—\([\gamma-\text{P}]\)ATP and \([\alpha-\text{P}]\)ADP were obtained from NEN Life Science Products. Pyridine, chloroform, methanol, and 88% formic acid were from Fisher. Triton X-100 was Surfact-Amps grade from Pierce. ACP was purchased from Sigma. Hybond-N nylon membranes were obtained from Amersham Pharmacia Biotech. PhosphorImager screens were from Molecular Dynamics. 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, Amersham Pharmacia Biotech. Formamide, salmon sperm DNA, and RNA standards were obtained from Life Technologies, Inc. All other chemicals were purchased from Sigma.

**Bacterial Strains and Growth Conditions**—Strains used in this study are derivatives of *E. coli* K-12 wild-type W3110, obtained from the *E. coli* Genetic Stock Center, Yale University. Strain MLK53 harbors a Tn10 insertion in the *lipL* (htrB) gene (18, 19). In some experiments, strain MC1000 (*lpxL* *lpxM* *lpxP*) (72) was used as the host for the vector pACYC184 (cam tetr p15A replicon) (73), or for the hybrid plasmid pSK57, which harbors *papA* on a 5.6-kilobase pair EcoRI fragment derived from Kohara *λ* clone 10D3 (74) in pACYC184. Cultures were generally grown in Luria-Bertani (LB) broth consisting of 10 g of NaCl, 5 g of yeast extract, and 10 g of Tryptone per liter (23). Tetracycline was used at a concentration of 50 μg/ml. 

**Isolation and Preparation of Lipid Substrates**—Lipid IV₃ and (Kdo)₂-lipid IV₆ were isolated and purified as described (14, 24, 25). (Kdo)₂-[\(\gamma\text{-}
\text{P}\)]lipid IV₆ was prepared from [\(\gamma\text{-}
\text{P}\)]lipid IV₅ as described previously (25–27), except that membranes of the 4'-kinase overproducing strain BLR(DE3)pLysS/pJK2 (28) were used to increase the yield of [\(\gamma\text{-}
\text{P}\)]lipid IV₅. Lauroyl, myristoyl, palmitoyl, R-3-hydroxymyristoyl, and palmitoleoyl-ACP were synthesized from the corresponding fatty acids and commercial acyl carrier protein, using solubilized membranes from the acyl-ACP synthase overproducing strain *E. coli* LCH109/pLCH5/pGP1–2, as described previously (10, 29).

**Assay for Palmitoleoyl Transferase Activity**—Cell-free extracts were assayed for palmitoleoyl transferase under conditions similar to those used for the lauroyl transferase (9, 10). The reaction mixture (typically 20 μl) contained 50 mM Hepes, pH 7.5, 0.1% Triton X-100, 5 mM MgCl₂, 250 μM [\(\gamma\text{-}
\text{P}\)]ATP, 5 μM (Kdo)₂-[\(\gamma\text{-}
\text{P}\)]lipid IV₅, and 32,000 cpm of [\(\gamma\text{-}
\text{P}\)]ATP. Palmitoleoyl-ACP was included as the acyl donor at 12 μM, and the final concentration of enzyme was 0.1 mg/ml of crude cell free extract. The tubes were incubated at 12 °C for 5–60 min with MC1000/pSK57 (lipXp⁺) or for 10–20 h with W3110 (wild-type) or MLK53 (*lpxL* mutant). Incubation at 12 °C improved the stability of the palmitoleoyl transferase and eliminated an interfering acylation of (Kdo)₂-[\(\gamma\text{-}
\text{P}\)]lipid IV₅ in crude extracts that was dependent upon the presence of palmitoleoyl-ACP. The reaction was stopped by spotting 5 μl of the assay mixture onto a silica gel thin layer plate. After the spots were allowed to dry under a cool air stream, the plates were developed in the solvent chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v/v), dried, and then exposed to a PhosphorImager screen for about 16 h. The percent conversion of substrate to product was determined using a Molecular Dynamics PhosphorImager, and the specific enzymatic activity was expressed as the ratio of contaminating free nucleotides by using a NucTrap probe purification column (Stratagene). Commercial RNA standards were analyzed in parallel during electrophoresis (34) and were stained with methylene blue to estimate the size of the lipXp transcript. The dried Northern blot was visualized using a PhosphorImager.

**RESULTS**

**A Novel Palmitoleoyl Transferase in Extracts of Cold-shocked Cells of *E. coli***—In *E. coli* or *S. typhimurium* cells cultured below 15 °C, a palmitoleate residue replaces most of the laurate that is normally linked to lipid A in cells grown at 30–42 °C (20, 21). Conversely, no palmitoleate is detected in lipid A of cells grown above 30 °C (8, 20, 21). We therefore assayed extracts of cells that had been shifted from 30 °C to 12 °C for 2 h during mid-log phase for the induction of a palmitoleoyl-ACP-dependent acyltransferase capable of using the key precursor (Kdo)₂-lipid IV₅ as the substrate (Fig. 1). The latter was previously shown to function as the acceptor for a laurate residue (Fig. 1) in the reaction catalyzed by the Kdo-dependent acyltransferase lipXpLHtrB in extracts of cells grown at 30 °C or higher (10). As demonstrated in Fig. 2, extracts (0.1 mg/ml) of wild-type *E. coli* shifted to 12 °C for 2 h converted (Kdo)₂-[\(\gamma\text{-}
\text{P}\)]lipid IV₅ to a more rapidly migrating product in the presence of palmitoleoyl-ACP, as judged by TLC analysis (Fig. 2), indicative of palmitoleate transfer. However, no acylated product was formed in the absence of palmitoleoyl-ACP in such cold-shocked extracts. Very little palmitoleoyl-ACP-dependent acylation of (Kdo)₂-[\(\gamma\text{-}
\text{P}\)]lipid IV₅ was detected in extracts of the 30 °C grown control cells (Fig. 2).

The slow assay temperature (12 °C) was necessary for demonstrating the induction of the palmitoleoyl transferase because of its instability at higher temperatures. In addition, the presence at 30 °C of an unrelated, interfering enzyme that transfers a palmitate residue from the 1-position of phosphatidylethanolamine or phosphatidylglycerol to (Kdo)₂-[\(\gamma\text{-}
\text{P}\)]lipid IV₅ or other lipid A precursors (35) obscures the palmitoleoyl-ACP-dependent acylation activity (data not shown). The ACP-independent palmitoyl transferase reaction is suppressed by carrying out the assays at 12 °C (data not shown).

**The Palmitoleoyl Transferase Is Not Encoded by the lipX Gene**—To determine if the cold-induced activity was dependent upon the lipX gene, we assayed the palmitoleoyl transferase activity in extracts of cold-shocked cells of strain MLK53 (17), in which the lipX gene is inactivated by an insertion mutation.

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[3] R. Bishop and C. R. H. Raetz, unpublished data.
As in the isogenic wild-type strain, palmitoleoyl-ACP-dependent acylation of (Kdo)₂-lipid IVₐ was induced in MLK53 cells grown at 12 °C (Fig. 3A). Very little palmitoleoyl-ACP-dependent acylation was observed in assays of extracts made from MLK53 cells grown at 30 °C, but the low level of activity seen in such extracts does appear to be slightly higher than the

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**Fig. 1. Role of (Kdo)₂-lipid IVₐ as a precursor of lipid A in normal and cold-shocked E. coli.** The intermediate (Kdo)₂-lipid IVₐ is a key precursor of lipid A, conserved in diverse Gram-negative bacteria (3). In cells grown at 30 °C or higher, (Kdo)₂-lipid IVₐ is acylated by the sequential action of LpxL and LpxM, which incorporate laurate and myristate, respectively, but do not function on precursors lacking the Kdo disaccharide (9–11). Palmitoleoyl-ACP-dependent acylation of (Kdo)₂-lipid IVₐ, catalyzed by the homologue LpxP, is observed only in extracts of cells subjected to cold shock (i.e. transferred from 30 °C to 12 °C for at least 15 min). Mass spectrometry demonstrates that ~80% of the lipid A moieties isolated from 12 °C grown E. coli cells contain palmitoleate instead of laurate. In 30 °C grown cells no palmitoleate is detected (20). Prior to elucidation of its function in lipid A biosynthesis (9–11), the lpxL gene was called htrB (high temperature requirement for rapid growth above 32 °C) (17, 18), and lpxM was designated mshB (multicopy suppressor of htrB) (19). The lpxP gene (which displays even greater sequence homology to lpxL than does lpxM) was provisionally termed ddg (sequenced in the GenBank™/EBI Data Bank with accession number 18722077), because of a proposed dam-dependent growth phenotype (69), but this idea was not confirmed (K. R. Sreekumar, unpublished data). The lpxL and lpxM genes have also recently been designated waaM and waaN by some authors (70, 71). We suggest that the waa nomenclature be restricted to bacterial glycosyltransferase genes (71), and that lpx be used for genes encoding enzymes of lipid A biosynthesis (3).
wild-type controls (Fig. 3A). The absence of the lauroyl transferase in extracts of MLK53 cells grown either at 12 °C or 30 °C was confirmed (Fig. 3B), consistent with previous studies (10). Therefore, the cold-induced palmitoleoyl transferase activity is independent of the lpxL gene. Cold shock also had very little effect on the activity of the lauroyl transferase in extracts of wild-type cells, which were assayed at 30 °C with lauroyl-ACP and (Kdo)2-[4-32P]lipid IVA as substrates (Fig. 3B).

Multiple Copies of lpxP, an E. coli Homologue of lpxL, Direct the Overexpression of the Cold-induced Palmitoleoyl Transferase—E. coli contains two genes that display sequence similarity to lpxL. One of these, previously designated msbB (now termed lpxM as shown in Fig. 1), encodes the myristoyl transferase that is required for the final acylation of lipid A in wild-type cells (11, 19). The other lpxL homologue, which was found in the E. coli genome (22) and is now designated lpxP (Fig. 1), codes for a protein that displays 54% identity and 73% similarity to LpxL (Fig. 4) over the entire 306-amino acid residue length of LpxL. To determine if lpxP encodes the cold shock-induced palmitoleoyl transferase activity, we assayed the palmitoleoyl transferase in extracts of MC1000(pSK57/lpxP1), which contains lpxP behind its native promoter on a pACYC184 vector, after shifting cells from 30 °C to 12 °C for 2 h. As shown in Fig. 3A, the palmitoleoyl transferase specific activity was about 7-fold higher in extracts of MC1000(pSK57/lpxP1) than in wild-type. However, the specific activity of the lauroyl transferase (LpxL) in extracts of MC1000(pSK57/lpxP1) was about the same as that in wild-type extracts (Fig. 3B), and the lauroyl transferase was not induced by cold shock.

Given the sequence similarity of LpxL and LpxP, together with the striking increase in the cold-induced palmitoleoyl transferase (Fig. 3A) in MC1000(pSK57/lpxP1) extracts, we propose that lpxP is the structural gene for the palmitoleoyl transferase.

Selectivity of the Cold-induced Acalytransferase for Palmitoleoyl-ACP—MLK53 (which lacks the lauroyl transferase) (10) was grown either at 30 °C or 12 °C, and extracts were then assayed with various substrates at 12 °C to determine the acyl chain specificity of the cold-induced palmitoleoyl transferase. As shown in Fig. 7, palmitoleoyl-ACP was the only acyl donor.
capable of supporting robust acylation of \((\text{Kdo})_2\cdot[4^\text{32P}]\text{lipid IV}_A\). Lauroyl-ACP, myristoyl-ACP, palmitoyl-ACP, \(R\)-3-hydroxymyristoyl-ACP, palmitoleoyl-coenzyme A, and palmitoyl-coenzyme A were virtually inactive as substrates (Fig. 7). A small amount of acylation was seen with myristoyl-ACP (Fig. 7), but this was not cold shock-induced, and might be explained by the direct acylation of \((\text{Kdo})_2\cdot[4^\text{32P}]\text{lipid IV}_A\) by LpxM.

**Fig. 4.** Sequence alignment of the \(\text{lpxL}(\text{htrB})\) and the \(\text{lpxP}\) gene products. \(\text{LpxL}\) and \(\text{LpxP}\) are each 306 amino acid residues long. The numbering refers to the sequence of \(\text{LpxL}\). \(\text{LpxL}\) and \(\text{LpxP}\) share 54% identity and 73% similarity, with one gap over a segment of 301 amino acids, as determined by BLASTp analysis (39).

**Fig. 5.** An assay for the cold shock-induced palmitoleoyl transferase. The time course (A) at 0.08 mg/ml crude extract protein and the protein concentration dependence (B) at 30 min of palmitoleoyl transfer from palmitoleoyl-ACP to \((\text{Kdo})_2\cdot[4^\text{32P}]\text{lipid IV}_A\) at 12 °C were determined using extracts of cold-shocked cells of MC1000(pSK57/lpxP\(^1\)), prepared as in Fig. 3.

**Fig. 6.** Kdo dependence of the cold shock-induced palmitoleoyl transferase. Crude extracts of MC1000(pSK57/lpxP\(^1\)), grown to mid-log phase and shifted for 2 h to 12 °C as in Fig. 3, were assayed at 0.1 mg/ml for palmitoleate transfer to the indicated acceptor under the standard assay conditions for 5 h at 12 °C. The acyl acceptor was either 6 \(\mu\text{M} \ (\text{Kdo})_2\cdot[4^\text{32P}]\text{lipid IV}_A\) or 6 \(\mu\text{M} \ [4^\text{32P}]\text{lipid IV}_A\), as indicated.

coenzyme A were virtually inactive as substrates (Fig. 7). A small amount of acylation was seen with myristoyl-ACP (Fig. 7), but this was not cold shock-induced, and might be explained by the direct acylation of \((\text{Kdo})_2\cdot[4^\text{32P}]\text{lipid IV}_A\) by LpxM.
LpxM normally prefers \((\text{Kdo})_2-[4^{-32}\text{P}](\text{lauroyl})-\text{lipid IV}_A\) as the acceptor (Fig. 1), but it can also function with \((\text{Kdo})_2-[4^{-32}\text{P}]\text{lipid IV}_A\) at a slow rate (11). The LpxM gene is intact in strain MLK53 (11). Taken together, the results clearly demonstrate that the cold-induced palmitoleoyl transferase is highly selective for palmitoleoyl-ACP, consistent with the effects of cold shock on the fatty acid composition of lipid A (Fig. 1).

**Time Course of Palmitoleoyl Transferase Induction following Cold Shock**—Cells were grown at 30 °C to mid-log phase, and were then divided into two equal portions, so that parallel cultures could be studied at 12 °C and 30 °C, as shown in Figs. 8 and 9. Samples were then removed from the cultures growing at 30 °C or 12 °C at 0.25, 0.5, 1, 2, 4, and 6 h. Over this time frame, the cold-shocked cells did not increase in density, as shown for the wild-type cells in Fig. 8, but slow growth did resume after about 8 h at 12 °C (data not shown). Extracts of the samples taken from both the 30 °C and the 12 °C cells were assayed for palmitoleoyl-ACP-dependent acylation of \((\text{Kdo})_2-[4^{-32}\text{P}]\text{lipid IV}_A\) under the palmitoleoyl transferase conditions for 11 h at 12 °C with the indicated acyl donors. The abbreviations are as follows: \(12:0\text{ACP}\), lauroyl-ACP; \(14:0\text{ACP}\), myristoyl-ACP; \(16:0\text{ACP}\), palmitoyl-ACP; \(16:1\text{ACP}\), palmitoleoyl-ACP; \(16:0\text{CoA}\), palmitoleoyl coenzyme A; \(16:1\text{CoA}\), palmitoyl coenzyme A.

**FIG. 7.** Acyl donor specificity of the cold shock-induced acyltransferase. Crude extracts of MLK53 (\(lpxL^{-}\)), grown to mid-log phase at 30 °C or shifted for 2 h to 12 °C as in Fig. 3, were assayed at 0.1 mg/ml for acylation of \((\text{Kdo})_2-[4^{-32}\text{P}]\text{lipid IV}_A\) under the palmitoleoyl transferase conditions for 11 h at 12 °C with the indicated acyl donors. The abbreviations are as follows: \(12:0\text{ACP}\), lauroyl-ACP; \(14:0\text{ACP}\), myristoyl-ACP; \(16:0\text{ACP}\), palmitoyl-ACP; \(16:1\text{ACP}\), palmitoleoyl-ACP; \(16:0\text{CoA}\), palmitoleoyl coenzyme A; \(16:1\text{CoA}\), palmitoyl coenzyme A.

**FIG. 8.** Growth of the wild-type \(E. coli\) strain W3110 at 30 °C and after a shift to 12 °C. The cells were grown to \(A_{600} = 0.4\) at 30 °C, and the culture was split into two equal portions at time 0. **Closed circles** indicate the \(A_{600}\) measurements for the culture held at 30 °C, and **open symbols** are the \(A_{600}\) measurements for the cells shifted to 12 °C at time 0.

Very little palmitoleoyl transferase was seen in extracts of the cultures held at 30 °C (Fig. 9), which gradually entered stationary phase over the course of the experiment. However, a small amount of palmitoleoyl transferase above background was observed in extracts of log phase MLK53 cells at 30 °C (Fig. 9B). This trace of palmitoleoyl transferase activity may account for the fact that MLK53 does actually synthesize some pentac- and even some hexa-acylated lipid A species (despite the absence of \(LpxL\)) at 30 °C, as noted previously in studies of lipid A composition (11, 36). Mass spectrometry (data not shown) indicates that palindrome indeed accounts for the presence of the penta-acylated lipid A species in MLK53 grown at 30 °C. It may be that when \(LpxL\) is missing the \(lpxP\) gene is switched on at higher temperatures than in wild-type cells.

\(lpxP\) mRNA is measurable in cells grown at 12 °C, but not at 30 °C—To determine if an increase in \(lpxP\) mRNA levels...
could account for the induction of the palmitoleoyl transferase during cold shock, Northern blotting was used to compare the \( \text{lpxP} \) mRNA levels extracted from cells grown at 30 °C or 12 °C. A blot of 10-\( \mu \)g RNA samples from both W3110 and MLK53, grown either at 30 °C or 12 °C, is shown in Fig. 10. A heavy band is detected at the position expected for \( \text{lpxP} \) mRNA (1000 nucleotides) in the total RNA from the cultures grown at 12 °C. There is no \( \text{lpxP} \) band in the RNA samples extracted from the cultures grown at 30 °C. The accumulation of \( \text{lpxP} \) mRNA in the cultures grown only at 12 °C (Fig. 10) and the time course of palmitoleoyl transferase induction at 12 °C (Fig.

**DISCUSSION**

As demonstrated previously in our laboratory, the enzyme LpxL(HtrB) catalyzes the transfer of laurate from lauroyl-ACP to the lipid A precursor (Kdo)\(_2\)-lipid IV\(_A\) in extracts of cells grown at 30 °C or above (Fig. 1) (10). We now have discovered an additional Kdo-dependent acyltransferase (LpxP) in *E. coli* that is required for the biosynthesis of a distinct molecular species of lipid A, present only in cells grown at low temperatures (Fig. 1) (20, 21). LpxP transfers palmitoleate from palmitoleoyl-ACP to (Kdo)\(_2\)-lipid IVA, and it is induced within 15 min in log phase cells shifted from 30 °C to 12 °C (Fig. 9). The palmitoleoyl transferase is a distinct enzyme, not an additional activity associated with the lauroyl transferase, as demonstrated by the fact that the palmitoleoyl transferase is induced normally (Fig. 3) in mutant cells (MLK53) (10) harboring an insertion in the \( \text{lpxL} \) gene. Furthermore, the palmitoleoyl transferase is greatly overproduced in strains harboring multiple copies of the \( \text{lpxP} \) gene expressed from its own promoter (Fig. 3). LpxL and LpxP share 54% identity and 73% similarity, with one gap over a segment of 301 out of 306 amino acids (Fig. 4), as determined by BLASTp analysis (39). In contrast, LpxL and LpxM (Fig. 1) show only 29% identity and 46% similarity, with 21 gaps over a sequence of 309 amino acids out of the 323 residues that comprise LpxM (39).

Extracts of wild-type *E. coli* grown at 12 °C contain both the lauroyl and the palmitoleoyl transferase activities (Fig. 3). The mechanisms by which the cells determine the amount of palmitoleate versus laurate that is transferred to (Kdo)\(_2\)-lipid IV\(_A\) at 12 °C in vivo may be quite interesting and will require further study. Based on mass spectrometry, we estimate that about 80% of the lipid A residues of cells grown at 12 °C are acylated with palmitoleate rather than laurate.\(^4\)

\(^4\) S. M. Carty and C. R. H. Raetz, unpublished data.
The level of lpxP mRNA increases by several orders of magnitude in cells grown at 12 °C (Fig. 10). This phenomenon must surely account for the induction of the palmitoleoyl transferase activity upon cold shock (Figs. 3 and 9). The time course of LpxP induction and its gradual decline in wild-type cells after several hours of growth at 12 °C (Fig. 9) is reminiscent of the response seen with many cold shock proteins, including the extensively studied RNA-binding protein CspA (37, 38, 40). The mechanisms by which cold shock induces CspA are still not fully understood (37, 38). Initial work suggested that increased transcription accounted for elevated CspA message levels in cold-shocked cells (37, 38). More recent studies support the view that CspA mRNA stability is also selectively enhanced at lower growth temperatures, possibly because of temperature effects on mRNA secondary structure (41, 42). RNAse E may play a direct role in the control of mRNA stability during cold shock (43). In addition, translation of cold shock mRNA may be selectively enhanced (41, 42). Given these considerations, it will be interesting to express lpxP behind a constitutive promoter and to measure LpxP activity in such constructs as a function of temperature. Expression of a reporter gene like lacZ or cat at various temperatures behind the native lpxP promoter might also be informative. Finally, a search for proteins that control the activity of the lpxP promoter in vivo might reveal how cells detect cold shock and readjust their lipid A pathway accordingly.

Among the enzymes of lipid A biosynthesis, LpxP may be unique in its induction at low temperatures, but it is not the only enzyme of the pathway that is regulated. The deacetylase (LpxC) that catalyzes the second, committed step of lipid A biosynthesis is increased about 10-fold in cells treated with the specific deacetylase inhibitor, L-573,655 (44, 45), or in point mutants with low levels of lpxA (44, 46). Deacetylase regulation is not accompanied by significant changes in lpxC mRNA (44). Instead, LpxC protein levels may be controlled by the membrane-associated protease FtsH (47).

Additional regulation of lipid A biosynthesis has recently been discovered by Miller and co-workers (48–50). These investigators have shown that the PhoP/PhoQ two component system is required for the modification of S. typhimurium lipid A with palmitate, L-4-aminoarabinoside, and/or 2-hydroxy-9-myrystate (48–50). While these modifications are not required for cell growth, they are critical for intracellular survival and resistance to basic antibacterial peptides (48–50). The PhoP/PhoQ system exerts its actions by regulating a second two component system, known as PmrA/PmrB (48, 49), which in turn is thought to control the expression of the putative enzymes that modify S. typhimurium lipid A. The PhoP/PhoQ system is switched on by low pH, as might be encountered by bacteria within endosomes, and by low magnesium ion concentrations (48). Whether or not the PhoP/PhoQ system is involved in the cold shock-induced modification of lipid A remains to be determined.

Both procaryotic (51–55) and eucaryotic (55–57) organisms increase the degree of unsaturation of their glycerophospholipids at low temperatures in a process termed homeoviscous adaptation (53), presumably to maintain the optimal physical properties of lipid A and its precursors (60–62). We have constructed a mutant in which the E. coli lpxP gene is inactivated. Although this strain can grow at 12 °C, it is extremely hypersensitive to antibiotics at low temperatures.2

To our knowledge, no one has previously reported an acyltransferase induced under conditions of cold shock (or other environmental stress) that selectively incorporates a specific fatty acyl chain into a membrane lipid precursor. The glycerol-3-phosphate acyltransferases of E. coli certainly are not regulated in this manner (52) and do not display a high degree of selectivity for their acyl donor substrates (63). There is only a slight difference in the fatty acid composition of the glycerolphospholipids of E. coli cells grown at 27 °C versus 37 °C (54). However, a 2-fold increase in unsaturation (mainly increased cis-vaccenate at the expense of palmitate) is observed in the glycerolphospholipids of E. coli cells grown at 17 °C versus 37 °C (54). The condensing enzyme (3-ketoacyl-ACP synthase II), encoded by the fabF gene, is thought to control the unsaturated acyl chain content of the E. coli glycerolphospholipids (52, 64). This may be mediated by a direct effect of temperature on FabF activity, which is higher at low temperatures and therefore might increase the size of the unsaturated fatty acid precursor pool appropriately (52, 64). In other organisms, including Synchocystis, Bacillus subtilis, Acanthamoeba, and carp, cold shock induces the transcription of specific fatty acid desaturases (55, 65, 75), likewise increasing the pool of available unsaturated fatty acids.

Mutants of E. coli that are defective in the fabA or the fabB genes require unsaturated fatty acids for growth (51, 52, 56, 66). Many different unnatural and polyunsaturated fatty acids, including trans-unsaturated compounds, can support the growth of such auxotrophs (51, 52, 56, 67). Unlike the lipid A acyltransferases (3), the glycerolphospholipid acyltransferases are not specific for their acyl donor substrates (63), and therefore many different unnatural fatty acid analogs can be incorporated into the glycerolphospholipids of such auxotrophs. The composition of lipid A in fatty acid auxotrophs supplemented with analogs has never been examined, but it should be unaffected, since exogenous fatty acids are activated as coenzyme A thioesters, and not as acyl-ACPs (1, 3, 66). However, DiRienzo and Inouye (68) reported that unsaturated fatty acid auxotrophs supplemented with elaidate (an unnatural trans-unsaturated fatty acid) do not grow at low temperatures and accumulate lipid A precursor(s). The chemical structures of these substances were not determined (68). Their characterization might yet provide interesting new insights into the regulation of lipid A biosynthesis and into the biological significance of lipid A modification with palmitoleate during cold shock.

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