Mutations in *firA*, Encoding the Second Acyltransferase in Lipopolysaccharide Biosynthesis, Affect Multiple Steps in Lipopolysaccharide Biosynthesis

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The product of the *firA* (*ssc*) gene is essential for growth and for the integrity of the outer membrane of *Escherichia coli* and *Salmonella typhimurium*. Recently, Kelly and coworkers (T. M. Kelly, S. A. Stachula, C. R. H. Raetz, and M. S. Anderson, J. Biol. Chem., 268:19866–19874, 1993) identified *firA* as the gene encoding UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase, the third step in lipid A biosynthesis. We studied the effects of six different mutations in *firA* on lipopolysaccharide synthesis. All of the *firA* mutants of both *E. coli* and *S. typhimurium* examined had a decreased lipopolysaccharide synthesis rate. *E. coli* and *S. typhimurium* strains defective in *firA* produced a lipid A that contains a seventh fatty acid, a hexadecanoic acid, when grown at the nonpermissive temperature. Analysis of the enzymatic activity of other enzymes involved in lipid A biosynthesis revealed that the *firA* mutations pleiotropically affect lipopolysaccharide biosynthesis. In addition to that of UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase, the enzymatic activity of the lipid A 4′ kinase (the sixth step of lipid A biosynthesis) was decreased in strains with each of the *firA* mutations examined. However, overproduction of FirA was not accompanied by overexpression of the lipid A 4′ kinase.

The *firA* gene of *Escherichia coli* and the analogous gene in *Salmonella typhimurium* have been shown to be essential for growth. The product of *firA* is important for maintaining resistance to hydrophobic antibiotics such as rifampin (19, 27). The outer membrane of gram-negative bacteria functions as a permeability barrier to hydrophobic antibiotics (30). For this reason, mutations affecting the synthesis of the outer membrane cause the cells to become hypersensitive to hydrophobic antibiotics (30). Recently, Kelly et al. (24) have demonstrated that *firA* encodes UDP-3-*O*-(*R*-3-hydroxymyristoyl)-glucosamine *N*-acyltransferase, the third committed step in lipid A biosynthesis. Studies with *S. typhimurium* indicate that the lipopolysaccharide produced in the *firA* mutant contains a seventh fatty acid, a hexadecanoic acid linked to the N-linked β-hydroxymyristate on the reducing end sugar (18). A mutation in *lpxB* (lipid A disaccharide synthase; see Fig. 1) also causes accumulation of the lipopolysaccharide precursor 2,3-diacyl-*N*-acetylglucosamine-1-phosphate (lipid X), some of which contains a hexadecanoic acid in the analogous position (termed lipid Y) (31). Lipid Y is not a substrate for the lipid A disaccharide synthase for further incorporation into lipopolysaccharide (23). 3-Deoxy-D-*manno*-octulosonate (KDO) cytidyltransferase activates KDO for the addition onto the lipid A precursor tetraacyl-disaccharide-1,4′-*bis*-phosphate (Fig. 1). Both *E. coli* and *S. typhimurium* grown in the presence of an inhibitor of this enzyme accumulate the lipid A precursor tetraacyl-disaccharide-1,4′-*bis*-phosphate (16). In *S. typhimurium*, much of this precursor tetraacyl-disaccharide-1,4′-*bis*-phosphate contains a hexadecanoate in a position analogous to that found in the lipopolysaccharide of the *firA* mutant strain of *S. typhimurium* (36). However, *E. coli* treated with the same drug does not significantly accumulate the hexadecanoic acid-

modified precursor of lipid A (16). In the present study, we identified the hexadecanoic acid-modified mature lipopolysaccharide in *E. coli* deficient in *firA*. The presence of heptaacyl lipid A in the lipopolysaccharide of *firA*-defective strains may indicate a block in lipid A biosynthesis or assembly. This block could cause accumulation of a precursor that can be modified with hexadecanoic acid and incorporated into the mature lipopolysaccharide. Alternatively, hexadecanoic acid acyltransferase activity may be increased by the stress of reduced lipopolysaccharide biosynthesis.

A defect in the lipid A 4′ kinase is expected to cause accumulation of a precursor in the cytoplasm that could be modified by hexadecanoic acid acyltransferase. In the present study, we found that all mutations in *firA*, the gene for UDP-3-*O*-acyl-glucosamine acyltransferase, reduce the activity of the lipid A 4′ kinase. However, overproduction of *firA* did not alter lipid A 4′ kinase activity.

Dicker and Seetharam (12) have proposed that FirA is important for RNA transcription. One mutation in the gene for an RNA polymerase subunit, *rpoB*, causes a synergistic increase in the temperature-sensitive phenotype of a *firA* mutation. In addition, FirA coprecipitates with RNA polymerase in immunoprecipitations of RNA polymerase from a strain overproducing FirA (12). Dicker and Seetharam (12) posited a catalytic requirement for FirA under certain conditions, as has been reported for ω protein, a protein thought to be physically involved in the modification of polymerase during the stringent response (22). The very low level of FirA in vivo is consistent with such a catalytic role. An interaction of the FirA protein with RNA polymerase could explain the pleiotropic effects of mutations in *firA*.

MATERIALS AND METHODS

Bacteria, plasmids, and media. The bacterial strains used are described in Table 1. Strain JCR4 was created by integration of pGAH317 (21), with a 1.5-kbp kanamycin resistance

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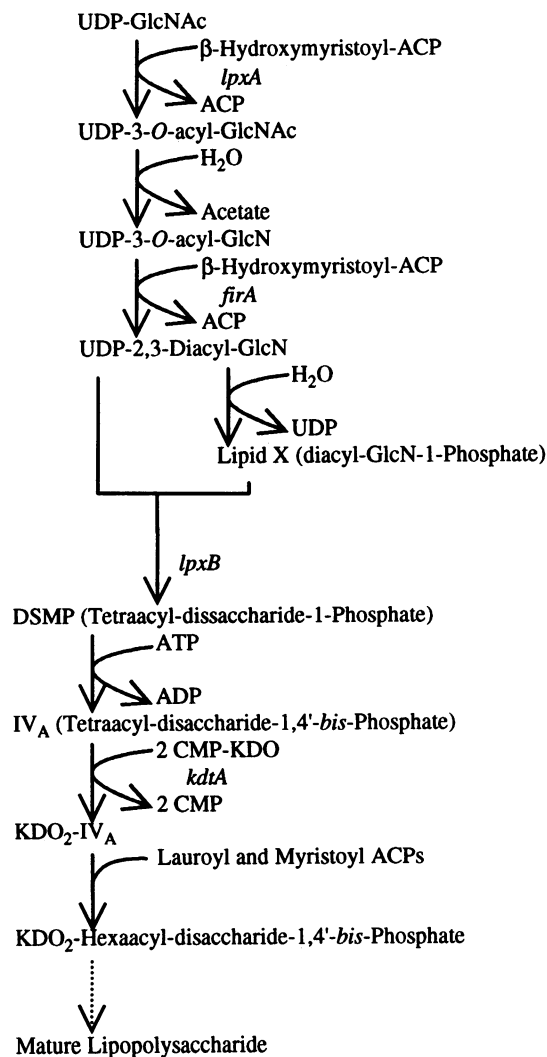
UDP-GlcNAc

β-Hydroxymyristoyl-ACP

lpxA

ACP

UDP-3-*O*-acyl-GlcNAc

H$_2$O

Acetate

UDP-3-*O*-acyl-GlcN

β-Hydroxymyristoyl-ACP

firA

ACP

UDP-2,3-Diacyl-GlcN

H$_2$O

UDP

Lipid X (diacyl-GlcN-1-Phosphate)

lpxB

DSMP (Tetraacyl-dissaccharide-1-Phosphate)

ATP

ADP

IV$_A$ (Tetraacyl-disaccharide-1,4'-*bis*-Phosphate)

2 CMP-KDO

kdtA

2 CMP

KDO$_2$-IV$_A$

Lauroyl and Myristoyl ACPs

KDO$_2$-Hexaacyl-disaccharide-1,4'-*bis*-Phosphate

Mature Lipopolysaccharide

FIG. 1. Pathway for the biosynthesis of lipid A. Evidence for this scheme has been presented previously (35). Genes *lpxA*, *lpxB*, *firA*, and *kdtA*, which are known to be involved in lipid A biosynthesis, are indicated. ACP, acyl carrier protein; GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine.

cassette inserted in a *Pst*I site in *hlpA*, into the chromosome of RL436 at 43°C (see Fig. 2). The temperature-sensitive mutation is in the upstream end of open reading frame 17 in RL436 (10a). pGAH317 does not contain the downstream portion of open reading frame 17; therefore, growth at 43°C is possible only after recombination of the plasmid into the chromosome. P1 transduction (38) was used to move the *hlpA*::Kan^r insertion into MF6R (*dapD*) with selection for the nearby *dapD*$^+$ gene and kanamycin resistance. At this point, the strain has two copies of *firA*, one from the plasmid and one from the chromosome. High concentrations of ampicillin enrich for cells that have excised the plasmid from the chromosome, to allow high-copy-number replication of the plasmid carrying the ampicillin resistance gene, *bla*. Excision of the plasmid leaves a single copy of *hlpA* and *firA* in the chromosome, with a 50% chance of having the kanamycin resistance insertion in the chromosome. A P1 lysate was made on this strain with the kanamycin resistance cassette left in the chromosome. This

mutation was transduced into MF6R/pMT*fir* with selection for *dapD*$^+$ and kanamycin resistance. The resulting strain was checked for loss of ampicillin resistance. The *recA56* mutation was introduced into the strain by Hfr mating with JC10240, selection for tetracycline resistance, and screening for UV sensitivity. The presence of a single copy of *hlpA-firA* with the Kan^r insertion in the chromosome of JCR4 was confirmed by Southern blotting (40).

The *E. coli* chromosomal portions of the plasmids used in this study are indicated in Fig. 2. pGAH317 has been described previously (21). p*Rsa* contains an *Rsa*I fragment encoding all of *firA* inserted into the *Sma*I site of pTZ18R (United States Biochemical Corporation). Plasmid pMT*fir* has the *Hin*dIII fragment containing *firA* from *E. coli* inserted into the *Hin*dIII site of vector pMT101 (17), which has a temperature-sensitive replicon. Plasmid pID2 (a kind gift from Ira Dicker) has been described previously (11). Plasmid pUCHS14-II contains the *S. typhimurium firA* gene and has been described previously (19).

All strains were grown in L broth (29) supplemented with diaminopimelic acid (50 μg/ml), ampicillin (100 μg/ml), tetracycline (10 μg/ml), kanamycin (50 μg/ml), or isopropyl-β-D-thiogalactopyranoside (1 mM) when appropriate.

Materials. Restriction enzymes were purchased from New England BioLabs, Beverly, Mass. Except as noted otherwise, chemicals were from Sigma, St. Louis, Mo. ^{32}P$_i$ was purchased from ICN, Irvine, Calif. UDP-*N*-acetyl[^{3}H]glucosamine and En^{3}Hance were from New England Nuclear. Kodak SB film was used for autoradiography. Glass-backed silica gel 60 thin-layer chromatography plates were from E. Merck, Darmstadt, Germany.

Rate of lipopolysaccharide biosynthesis. The rate of lipopolysaccharide synthesis was measured as previously described (14). Cells were grown in L broth at 30°C and then switched to 43°C for 1 h (8 h for JCR4) prior to pulse labeling with ^{32}P$_i$ for 5 min. Quantitation of ^{32}P$_i$ incorporation into lipopolysaccharide and glycerophospholipids (14) was done with a Molecular Dynamics (Sunnyvale, Calif.) Phosphorimager after exposure to Kodak storage phosphor screens. Data analysis employed the software supplied with the Phosphorimager in the Louisiana State University Biotechnologies Core Laboratory.

Preparation of cell extracts and enzyme assays. Cell extracts for enzyme assays were prepared from stationary-phase cells grown at 30°C (43°C for 8 h for JCR4 and its control) in L broth. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C. Cells were resuspended in 10 mM potassium phosphate (pH 7.0) and broken by passage through a French press at 18,000 lb/in^2. Unbroken cells were removed by centrifugation at 5,000 × *g* for 5 min. Membranes were removed by centrifugation at 4°C for 60 min at 210,000 × *g*. The membrane fraction was suspended in Tris-HCl (pH 8.0) for use in the lipid A 4' kinase assay. Protein concentration was determined by the bicinchoninic acid (Pierce Chemical Co., Rockford, Ill.) method of Smith et al. (39). Protein concentrations were made equal prior to enzyme assays.

The early steps of lipid A biosynthesis were assayed as described by Anderson and Raetz (1) by using UDP-*N*-acetyl[^{3}H]glucosamine or UDP-3-*O*-acyl[^{3}H]glucosamine as the initial substrate. Products were separated by using silica gel thin-layer chromatography developed with chloroform-methanol-acetic acid-water (25:15:4:2, vol/vol). Products were identified by comigration with known standards.

The lipid A disaccharide synthase was assayed as described by Crowell and coworkers (9). Quantitation was done with a Molecular Dynamics Phosphorimager.

The lipid A 4' kinase was assayed as described by Ray and Raetz (37), with [^{32}P]tetraacyl-disaccharide-monophosphate

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference(s) or source
E. coli		
MF6	_thr-1 ara-14 dapD4_ Δ(_gal-att_λ)_99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1_	15
MF6R	_thr-1 ara-14 dapD4_ Δ(_gal-att_λ)_99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1 rpoB_	8
SM105	_thr-1 ara-14_ Δ(_gal-att_λ)_99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1_	14
SM66	_thr-1 ara-14_ Δ(_gal-att_λ)_99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1 zae_::Tn_10 firA66_	14; this study
SM111	_thr-1 ara-14_ Δ(_gal-att_λ)_99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1 firA11_	14; this study
SM101	_thr-1 ara-14_ Δ(_gal-att_λ)_99 hisG4 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 eda-50 rfbD1 thi-1 lpxA2_	14
MN7	Δ(_gal-att_λ)_99 pgsA444 hisG4 gyrA283 rpsL136 xyl-5 mtl-1 lacY1 tsx-78 rfbD1 thi-1 lpxB1_	32
JM83	_ara_ Δ(_lac-proAB_) _rpsL_ (Str^r) [φ80 d_lac_ Δ(_lacZ_) M15]	46
JM_fir_	_ara_ Δ(_lac-proAB_) _rpsL_ (Str^r) (φ80 d_lac_ Δ(_lacZ_) M15) _zae_::Tn_10 firA66_ (Tet^r temperature-sensitive P1 transductant of JM83 with SM66 as donor)	This study
JMtet	_ara_ Δ(_lac-proAB_) _rpsL_ (Str^r) [φ80 d_lac_ Δ(_lacZ_) M15] _zae_::Tn_10_ (Tet^r temperature-resistant P1 transductant of JM83 with SM66 as donor)	This study
RL453	HfrP4X _metB firA53_(Ts^53)	26
RL436	HfrP4X _metB_(Ts^36) (in open reading frame 17)	26
JCR200	MF6R _dapD_^+ (temperature-resistant _dapD_^+ P1 transductant of MF6R with SM66 as donor)	This study
JC10240	HfrPO45 _recA56 srlA_::Tn_10 thr-300 ilv-318 rpsE300_	10
JCR4	JCR200 _recA54 srlA_::Tn_10 hlpA_::Kan^r (_firA_)/pMT_fir_	This study
JCR20	JCR200 _firA200_	8
JCR66	JCR200 _firA66_ (_dapD_^+ temperature-sensitive P1 transductant of MF6R with SM66 as donor)	This study
JCR111	JCR200 _firA111_ (_dapD_^+ temperature-sensitive P1 transductant of MF6R with SM111 as donor)	This study
JCR53	JCR200 _firA53_ (_dapD_^+ temperature-sensitive P1 transductant of MF6R with RL453 as donor)	This study
S. typhimurium		
SH5014	_ilv-1178 thr-914 his-6116 metA22 metE551 trpB2 xyl-404 H1-b H2-e, n, x flaA66 rpsL120 rfaJ4041_	41
SH7622	_ilv-1178 thr-914 his-6116 metA22 metE551 trpB2 xyl-404 H1-b H2-e, n, x flaA66 rpsL120 rfaJ4041 ssc-1_ (= _firA_)	19, 42

as the radiolabeled substrate. The enzyme preparations were 5 mg of total membrane protein per ml in Tris-HCl (pH 8.0; final concentration, 2 mg/ml). The enzyme assays were linear with both time and enzyme concentration for all of the strains used.

Antibiotic sensitivity. Antibiotic sensitivity was measured by the agar diffusion method of Bauer et al. (3) by using 1 mg of novobiocin on each disc. Antibiotic sensitivity was determined at 37°C on Luria-Bertani agar plates.

Purification of lipopolysaccharide. Lipopolysaccharide was purified by the hot phenol-water method of Westphal and coworkers (45). Samples were separated on sodium dodecyl sulfate (SDS)–15% polyacrylamide gel electrophoresis (PAGE) gels (20) and silver stained as described by Tsai and Frasch (43). Cells were grown for 8 h at 43°C (1 h for _S. typhimurium_ strains) before purification.

Purification of lipid A. Cells were grown for 8 h at 43°C in L broth. The bacteria were collected by centrifugation at 4,000 ×
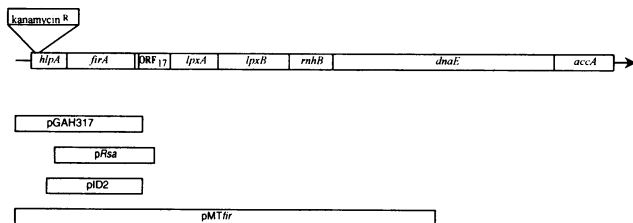

FIG. 2. Genetic organization of the min 4 region of the _E. coli_ chromosome. The site of insertion of the kanamycin resistance cassette in JCR4 is shown. Two genes, _rnhB_ (23) and _accA_ (28), have been identified since the publication of the most recent map of _E. coli_ (2). Below the chromosomal map are indicated the regions of the chromosome contained on the indicated plasmids. Plasmid pMT_fir_ is temperature sensitive for replication.
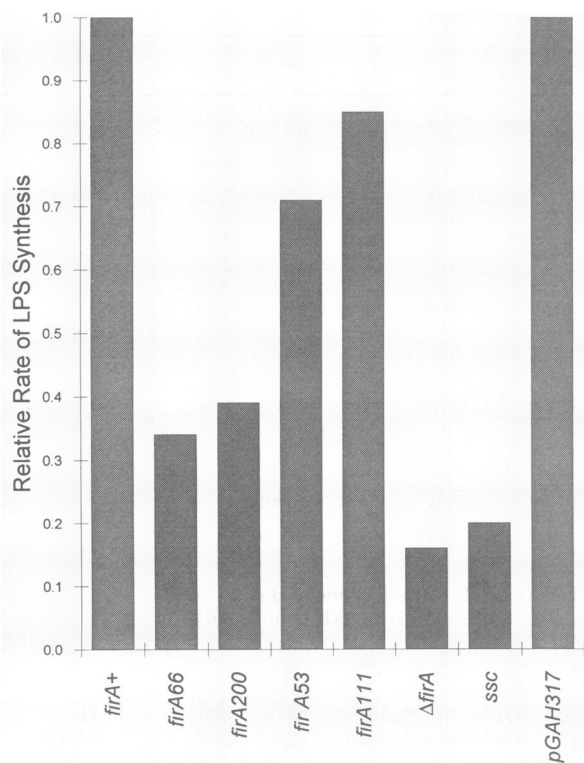
_g_ for 15 min. The cell pellets were washed once in 10 mM sodium phosphate (pH 7.0) and suspended in sodium phosphate, and 3.75 volumes of methanol-chloroform (2:1, vol/vol) was added. After 20 min at room temperature, the lipopolysaccharide was precipitated by centrifugation at 4,000 × _g_ for 15 min. The pellet was washed twice with 10 mM sodium phosphate-methanol-chloroform (0.8:2:1, vol/vol). The lipopolysaccharide-containing pellet was suspended in 1% acetic acid and then boiled for 90 min. After cooling, 2.2 volumes of chloroform-methanol (1:1) was added to form a two-phase Bligh and Dyer (5) extraction. The lower phase was washed twice with an acidic upper phase as described by Nishijima and Raetz (32). The lower phase was washed with 0.1 N HCl-methanol (1:2) and evaporated to dryness under nitrogen. This was suspended in chloroform-methanol (3:1). One hundredth of a volume of 1 M ammonia was added to raise the pH. This sample was used directly for mass spectral analysis.

Electrospray mass spectra. Electrospray mass spectra were generated with a model 201 single-quadrapole mass spectrophotometer (Vestec Corporation, Houston, Tex.) that was fitted with a 10-kV conversion dynode, had a 2,000 _m/z_ mass range, and was located in the Louisiana State University Biotechnologies Core Laboratory. Sample mixtures were infused by syringe pump (Orion Research) at a flow rate of 6 ml/min, with spectra acquired as single 5-s scans. The source block was heated to 250°C, the spraying chamber was at 55°C, the repeller voltage was 18 V, and the nozzle voltage was set at 230 V. Calibration of the mass spectrophotometer was performed prior to sample analysis, with 1-decanesulfonic acid, sodium salt.

Each spectrum is the average of more than 20 scans. High values (noise) were ignored if they differed by greater than 5 standard deviations from the mean for that _m/z_.

FIG. 3. Relative rate of lipopolysaccharide (LPS) biosynthesis. Bacteria were pulse-labeled with $^{32}P_i$ for 5 min. Lipid A and glycerophospholipids were quantitatively isolated (14), and the amount of radioactivity in each was determined. Values are expressed as the ratio of lipopolysaccharide to glycerophospholipids, normalized to the isogenic host strain for each sample. *firA66* is from strain SM66, *firA200* is from JCR20, *firA53* is from JCR53, *firA111* is from SM111, and Δ*firA* is the *firA*::Kan^r mutation in strain JCR4. All strains were grown at 43°C for 1 h prior to labeling (except JCR4, which was grown for 8 h at 43°C prior to labeling).

FIG. 4. Silver-stained SDS-PAGE of isolated lipopolysaccharides. The *S. typhimurium firA*$^+$ strain was SH5014, and the *S. typhimurium firA* strain was SH7622. The *E. coli firA*$^+$ strain was JMtet, and the *E. coli firA* mutant strain was JM*fir*.

RESULTS

Lipopolysaccharide production in *firA* mutants. Localized mutagenesis of the 4-min region of the *E. coli* chromosome produced a number of temperature-sensitive alleles of *firA* (14, 26). The *firA* gene from temperature-sensitive strains complemented with a plasmid that produces only FirA was transduced into strain MF6R to make isogenic strains (Table 1). We measured the rates of lipopolysaccharide biosynthesis in various *E. coli* and *S. typhimurium* strains by measuring the rate of incorporation of radiolabeled P_i into lipopolysaccharide (14). Figure 3 shows that cells with every mutant allele of *firA* tested had a reduced rate of lipopolysaccharide biosynthesis compared with wild-type levels. The rate of lipopolysaccharide biosynthesis was normalized to the rate of glycerophospholipid biosynthesis (14). We obtained similar results when the rate of protein biosynthesis was used for normalization (data not shown). The most severe inhibition of lipopolysaccharide biosynthesis was in strain JCR4. JCR4 produced no FirA from the chromosome because of an insertion in the *firA* operon upstream of the FirA coding sequence (Fig. 2). JCR4 contains plasmid pMT*fir*, which has a temperature-sensitive replicon and contains *firA*$^+$. JCR4 continued to grow at 43°C only when a second plasmid, such as pRsa, produced a normal FirA protein. Because it is not necessary to complement the essen-
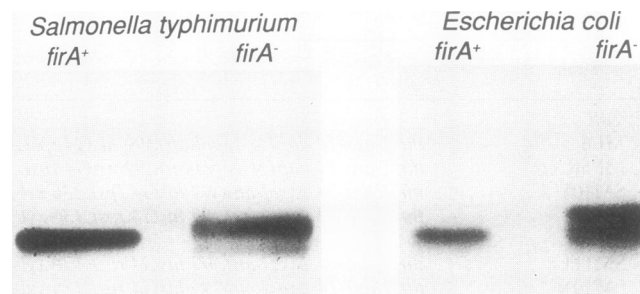
tial gene *lpxA* in JCR4, a second promoter must exist between *firA* and *lpxA*.

The above-described results confirm the finding of Kelly and coworkers (24) that *firA* is involved in lipopolysaccharide biosynthesis, but the results do not indicate whether *firA* is involved in multiple steps of lipid A biosynthesis.

***E. coli* produces altered lipopolysaccharide in the presence of mutant *firA*.** The isolated lipopolysaccharides from JCR4/pGAH317 (*firA*$^+$), JCR4 (*firA* mutant), SH5014 (*firA*$^+$), and SH7622 (*firA* mutant) were analyzed by SDS-PAGE (Fig. 4). As shown previously (18), the *S. typhimurium firA* mutant strain produced a more slowly migrating lipopolysaccharide. The *E. coli firA* mutant strain also produced a lipopolysaccharide with altered mobility. However, less than 50% of the *E. coli* lipopolysaccharide migrated at the slower rate in SDS-PAGE.

The slower migration of the lipopolysaccharide of *E. coli firA* mutant strains may be due to an additional fatty acid on the lipid A. Alternatively, multiple other modifications of lipopolysaccharide have been identified, such as additional phosphates or phosphorylethanolamine (35).

Electrospray mass spectrometry of lipid A preparations demonstrates the existence of heptaacyl lipid A in *E. coli*. Electrospray mass spectral analysis of the lipid A produced from *E. coli firA*$^+$ (JCR4/pGAH317) and the *firA* mutant (JCR4) indicates production of heptaacyl lipid A by JCR4 (Fig. 5). The lipid A for mass spectral analysis was isolated by using mild conditions (1% acetic acid), which are less likely to remove the acyl-oxy-acyl fatty acids than are the harsher hydrolysis conditions (0.2 N HCl) used previously (14, 18). This method of isolation of lipid A does hydrolyze some of the anomeric phosphate (Fig. 5). Both the *firA*$^+$ and *firA* mutant strains contain hexaacyl lipid A with a −1 (1798) or −2 (899) charge (Fig. 5). In addition, both samples have the hexaacyl lipid A that has lost one of the phosphates. Along with these bands, the *firA* mutant lipid A also contains molecules with a molecular mass corresponding to the addition of a hexadecanoate. This heptaacyl form is found with a −2 (1018) charge and a −1 charge with loss of a phosphate (1957). We did not detect pentaacyl forms of lipid A in these *E. coli* strains.

These results prove that *E. coli firA* mutant strains produce a heptaacylated lipid A. The identification of a lipid A molecule lacking a phosphate is most likely an artifact of the isolation procedure. Therefore, these results do not indicate or rule out the presence of an underphosphorylated lipid A, as would be expected from lowered production of the 4' kinase.

The above-described results and those of Helander and coworkers (18) indicate that a mutation in *firA* causes produc-
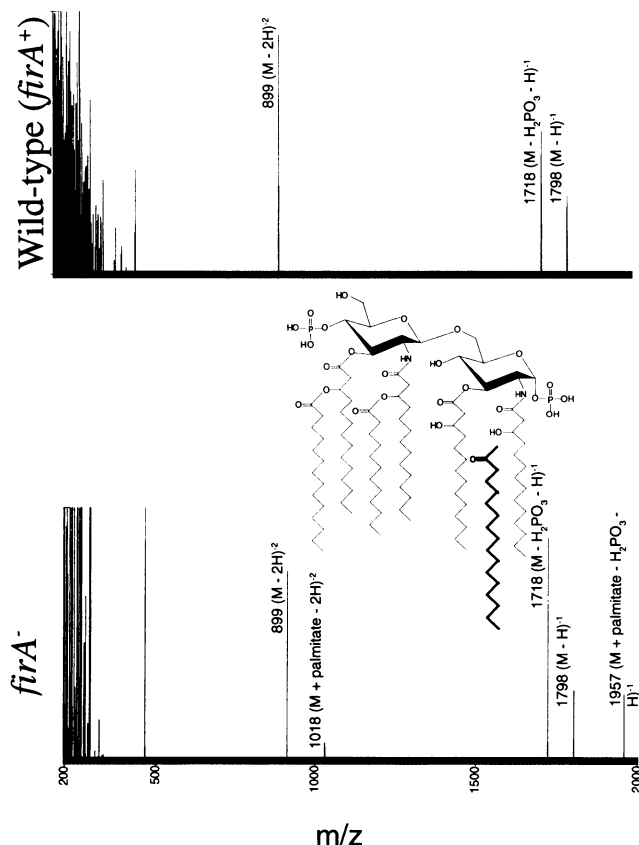
FIG. 5. Partial negative ion electrospray mass spectra of lipid A derivatives extracted from JCR4/pGAH317 ($firA^+$) and JCR4 ($firA$ mutant). The abscissa denotes the relative intensity of the given m/z. The inset shows the proposed structure of the lipid A produced by $firA$-defective strains. The hexadecanoic acid shown in boldface is found as a partial substituent in $firA$ mutant strains of *E. coli* but not in wild-type strains.

FIG. 6. Lipid A 4' kinase activity on [^{32}P]tetraacyl-disaccharide monophosphate. [^{32}P]tetraacyl-disaccharide monophosphate was incubated with membrane fractions from strains JMtet, JM*fir* ($firA200$), SH7622 ($ssc-1$; *S. typhimurium firA* mutant), SH5014 (*S. typhimurium* $firA^+$), and JM*fir*/pID2 ($firA$ overexpressed). Reactions occurred at 37°C for 5 and 10 min. Reaction products were separated by thin-layer chromatography on silica gel 60 plates in chloroform–pyridine–88% formic acid–water (40:60:16:5, vol/vol). Autoradiography was done at room temperature for 2 days. Abbreviations are as in Fig. 1.

tion of a heptaacyl lipopolysaccharide. Other mutations in lipid A biosynthesis that result in accumulation of lipid A precursors on the cytoplasmic side of the inner membrane accumulate modified precursors with the extra fatty acid hexadecanoic acid (31, 36). However, the hexadecanoic acid-containing precursor is not a substrate for the disaccharide synthase, the fifth step of lipid A biosynthesis. For this reason, lipid A precursors modified with a hexadecanoic acid prior to the disaccharide synthase (step 5 in Fig. 1) would not be expected to be incorporated into the lipopolysaccharide. Alternatively, the hexadecanoyl acyltransferase may be activated under conditions of stress. A specific assay of the disaccharide synthase indicated that the activity of this enzyme was unaltered in all of the *firA* alleles tested.

The lipid A 4' kinase is defective in all *firA* mutant strains. The lipid A 4' kinase adds a phosphate to the 4' position of tetraacyl-disaccharide-1-phosphate. In *E. coli*, this step is required for addition of the first core sugar, KDO (4). We determined the lipid A 4' kinase activities in strains with different alleles of *firA* (Fig. 6 and 7). Strains with every temperature-sensitive allele of *firA* ($firA66$, $firA200$, $firA53$, $firA111$, Δ$firA$, and *ssc*) had defective 4' kinase activity; however, strains overexpressing *firA* (JM*fir*/pGAH317, JM*fir*/pID2, and SH7622/pUCSH14-II) did not overexpress this activity. It should be noted that the $firA66$ mutation reduced the level of
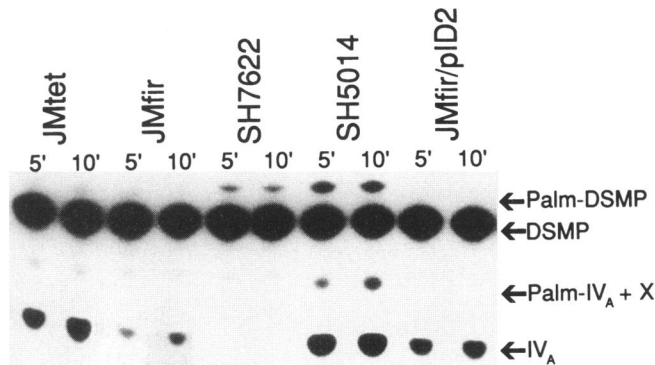
UDP-3-*O*-acylglucosamine acyltransferase to less than 1% of wild-type levels (data not shown).

The activity of the lipid A 4' kinase may be lowered because the *firA* mutant strains produce a substance that inhibits the lipid A 4' kinase. If the decrease in lipid A 4' kinase activity is due to an inhibitor being produced in *firA* mutant strains, the activity of the 4' kinase of a wild-type strain would be expected to be reduced if mixed with the potential inhibitor in vitro. When the membrane fraction from a $firA^+$ strain (SH5014) was mixed with the membrane of a *firA* mutant strain (SH7622), the resulting lipid A 4' kinase activity was equal to the sum of the enzyme activities from both strains. This suggests that the loss of activity was not due to an inhibitor of the lipid A 4' kinase found in the *firA* mutant cells.

Note that *S. typhimurium* strains produced additional products derived from tetraacyl-disaccharide-1-phosphate (Fig. 6). These additional lipids probably contain an additional hexadecanoic acid on the C-2 β-hydroxymyristate, as has been observed previously (6, 36). Helander and coworkers (18) have shown that the lipopolysaccharide produced from the *S. typhimurium firA* mutant strain contains this additional fatty acid. As seen in Fig. 6, the apparent activity of this hexadecanoate acyltransferase was not increased in the *firA* mutant strain.

The half-life of the lipid A 4' kinase is unaffected in *firA* mutant strains. One possible explanation for the decrease in 4' kinase activity is a reduction in 4' kinase enzyme stability in *firA* mutant strains. To determine whether this is the case, we incubated the membrane preparations (diluted to 5 mg/ml) at 42°C for 10, 60, 120, and 180 min. After incubation at 42°C, the activity of the 4' kinase was assayed as described previously. These experiments demonstrated that for *S. typhimurium* SH5014 ($firA^+$) and SH7622 ($firA$ mutant), the half-life of the lipid A 4' kinase is unaffected by the *firA* mutation. Both strains possess a lipid A 4' kinase with an in vitro half-life of 4.8 h at 42°C.

***firA* strains are hypersensitive to novobiocin.** Defects in the synthesis of the outer membrane cause the cell to become hypersensitive to hydrophobic antibiotics such as rifampin and novobiocin (30, 44). The activity of the lipid A 4' kinase does not correlate with the rate of synthesis of lipopolysaccharide in
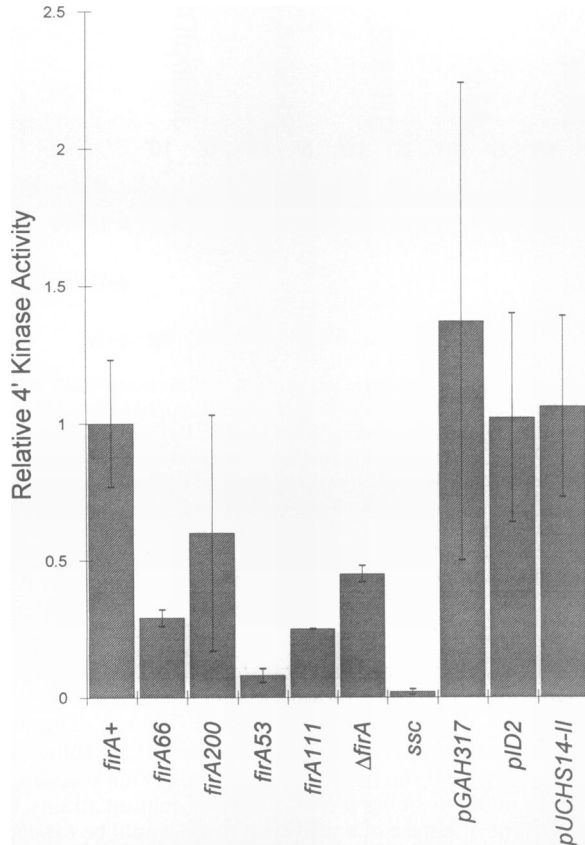
FIG. 7. Relative lipid A 4' kinase activity in strains with various alleles of *firA*. [^{32}P]tetraacyl-disaccharide monophosphate was incubated with membrane fractions as described in the legend to Fig. 6. The number of units of lipid A 4' kinase (nanomoles per minute per milligram of protein) was determined for each sample and then normalized to the number determined for the isogenic wild-type strain (the kinase value for the *firA*$^+$ strains is 1.00 by definition). Standard deviations are indicated. Plasmids pID2 (*E. coli*) and pUCHS14-II (*S. typhimurium*) overexpress *firA*, and plasmid pGAH317 overexpresses both *firA* and upstream gene *hlpA*. *firA66* is from JM*fir*, *firA200* is from JCR20, *firA53* is from JCR53, *firA111* is from JCR111, *ssc* is the *firA* mutation in *S. typhimurium* SH7622, and Δ*firA* is the *firA*::Kan^r mutation in JCR4 (after growth at 43°C for 8 h).

the different *firA* mutant strains (compare Fig. 3 and 7). To determine whether the sensitivity of *firA* mutant strains to hydrophobic antibiotics correlates with lipid A 4' kinase activity or lipopolysaccharide biosynthesis, we determined, by the method of Bauer et al. (3), the relative resistance of each strain to novobiocin. Figure 8 shows the zone of clearing, relative to that of the isogenic *firA*$^+$ strain, for each of the *firA* alleles. This demonstrates that resistance to hydrophobic antibiotics is directly related to the rate of lipopolysaccharide biosynthesis, with the notable exception of the *S. typhimurium firA* mutant (compare Fig. 3 and 8). The greater resistance of the *S. typhimurium firA* mutant strain may be due to the greater production of heptaacyl lipopolysaccharide. The strain background did not significantly alter the results of the antibiotic sensitivity assays when the results were expressed relative to the wild type. These data also demonstrate that mutations in two other genes known to be involved in lipopolysaccharide biosynthesis, *lpxA* and *lpxB*, increase sensitivity to novobiocin.
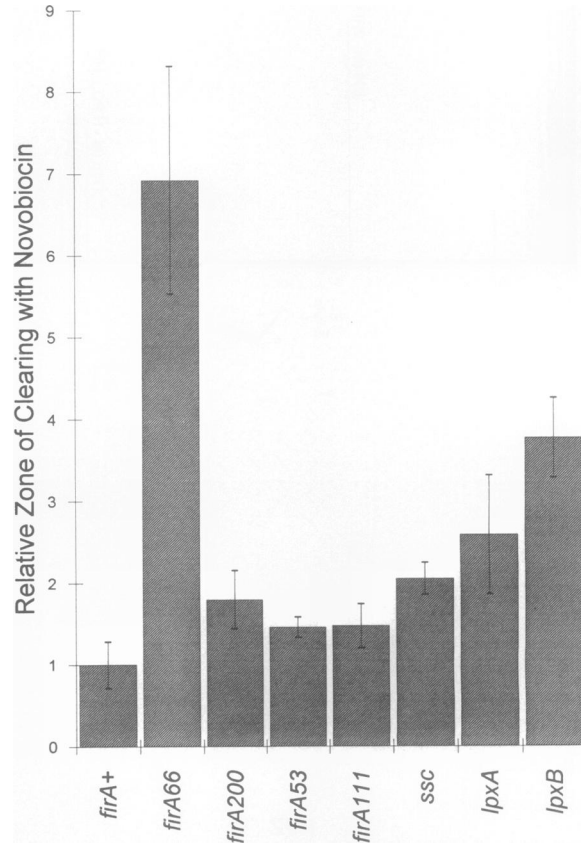

FIG. 8. Sensitivity of *firA* mutant strains to novobiocin. Each zone of inhibition was measured in millimeters from the edge of the novobiocin-containing filter disc to the edge of the clearing zone. All values are expressed as the ratio of the zone of inhibition of the given strain to that of the isogenic wild-type strain. The values shown are averages of at least four determinations. *firA66* is from SM66, *firA200* is from JCR20, *firA53* is from JCR53, *firA111* is from JCR111 and SM111, *ssc* is the *firA* mutation in *S. typhimurium* SH7622, *lpxA* is from SM101, and *lpxB* is from MN7.

DISCUSSION

The data presented in this study indicate that mutations in *firA* affect multiple steps in the synthesis and assembly of lipopolysaccharide. Although *firA* encodes UDP-3-*O*-acyl-GlcN acyltransferase (24), the *firA* mutants accumulate a heptaacyl lipid A at the nonpermissive temperature for growth. This is reminiscent of other lipid A biosynthesis mutants (6, 36). In these cases, a lipid A precursor remains on the cytoplasmic side of the inner membrane for a prolonged time. The enzyme responsible for the addition of a hexadecanoic acid to the lipid A precursor is most likely localized on the cytoplasmic surface of the inner membrane, where it modifies the accumulated precursor (6). Addition of a hexadecanoic acid to the lipid A of the *firA* mutants indicates the retention of a precursor in the cytoplasm for modification. Because the lipid A disaccharide synthase cannot use a hexadecanoic acid-modified substrate (34), this modification in the *firA* mutants must occur after this step (Fig. 1).

Clementz and Raetz (7) mapped and cloned the gene for the KDO transferase, *kdtA* (Fig. 1). *kdtA* maps to min 81 on the *E. coli* chromosome. The *firA* mutation did not significantly affect KDO transferase activity (data not shown). The only other

enzymes required for synthesis of a viable lipopolysaccharide are the lauroyl and myristoyl lipid A acyltransferases and the lipid A 4′ kinase. Qureshi and coworkers (33) have demonstrated the presence of a pentaacyl lipid A assembled in deep rough mutants of *E. coli*. Our analysis does not detect any lipid A lacking either a lauroyl or a myristoyl group. These results indicate that *firA* mutation does not cause a significant decrease in the activity of either of these two acyltransferases.

FirA may affect the transport of the lipid A molecule through the inner membrane. If this is the case, accumulation of lipopolysaccharide precursors in the cytoplasm may repress the production of lipid A biosynthetic enzymes. Retention of lipopolysaccharide on the cytoplasmic surface of the inner membrane would allow it to be a substrate for the lipid A hexadecanoic acid acyltransferase.

Our results show that the activity of the lipid A 4′ kinase is decreased in all of the *firA* mutations studied, in both *E. coli* and *S. typhimurium* (Fig. 7). Overproduction of the *firA* gene product, UDP-3-O-acyl-GlcN acyltransferase, does not increase the activity of the lipid A 4′ kinase. This indicates that the decrease of lipid A 4′ kinase activity is a secondary effect of the altered UDP-3-O-acyl-GlcN acyltransferase. UDP-3-O-acyl-GlcN acyltransferase may help to stabilize the active conformation of the lipid A 4′ kinase, or the *firA* mutations may cause repression of the gene for the lipid A 4′ kinase. If FirA does help to stabilize the active conformation of the lipid A 4′ kinase, the effect of the stabilization is not seen in the in vitro assays. FirA may interact with RNA polymerase to regulate the transcription of certain genes, as proposed by Dicker and Seetharam (12). The present studies do not answer the question of what causes the decrease in lipid A 4′ kinase activity. This question may be more easily answered once the lipid A 4′ kinase gene or protein has been identified. Reduced lipid A 4′ kinase activity may cause accumulation of a lipopolysaccharide precursor on the cytoplasmic side of the inner membrane that is a substrate for the lipid A hexadecanoic acid acyltransferase. Alternatively, the presence of heptaacyl lipid A may be a secondary result of some other stress on the cell in *firA*-defective strains.

firA mutations cause a much greater defect in UDP-3-O-acyl-GlcN acyltransferase activity than in lipid A 4′ kinase activity. For this reason, the precursor of the lipid A 4′ kinase, tetraacyl-disaccharide-1-phosphate, did not significantly accumulate in *firA* mutant cells at the nonpermissive temperature (data not shown).

The reduced rate of lipopolysaccharide biosynthesis at the nonpermissive temperature in *firA* mutant strains can explain the lethality of these mutations and the supersensitivity to hydrophobic antibiotics. The percentages of lipopolysaccharide biosynthesis reduction in severe *firA* mutants (84% reduction) and severe *lpxA* mutants (90% reduction) (14) are similar. The product of the *lpxA* gene catalyzes the first step in lipopolysaccharide biosynthesis (Fig. 1). Cells with the *lpxA* and *lpxB* mutations share with *firA* strains the hydrophobic-antibiotics-supersensitive phenotype (Fig. 8) (44). Accumulation of the heptaacyl lipopolysaccharide may also contribute to the temperature-sensitive phenotype of *firA* mutant strains by altering the conformation of the lipid A (25). The presence of heptaacyl lipid A is not likely to be the sole cause of the lethal phenotype, since *S. minnesota* is able to survive at 42°C with 70% heptaacyl lipid A (13, 18).

The presence of heptaacyl lipopolysaccharide may actually increase the resistance of bacteria to hydrophobic antibiotics. Although *firA* mutant *S. typhimurium* SH7622 had a much greater decrease in lipopolysaccharide biosynthesis than the corresponding *E. coli* strains (Fig. 3), SH7622 was more

resistant to the hydrophobic antibiotic novobiocin (Fig. 8). The difference in antibiotic susceptibility may be explained by the larger percentage of heptaacyl lipopolysaccharide found in the *S. typhimurium firA* mutant strain than in *E. coli firA* mutant strains (Fig. 5).

This work demonstrates that *E. coli* is capable of synthesizing heptaacyl lipopolysaccharide and that FirA is important for more than one step in the synthesis of lipid A. Ongoing work in our laboratory will better define the precise roles of FirA in lipopolysaccharide synthesis and assembly.

ACKNOWLEDGMENTS

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