Lipid A Modifications Characteristic of Salmonella typhimurium Are Induced by NH₄VO₃ in Escherichia coli K12*

DETECTION OF 4-AMINO-4-DEOXY-1-ARABINOSE, PHOSPHOETHANOLAMINE AND PALMITATE*

Zhimin Zhou‡, Shanhua Lin§, Robert J. Cotter§, and Christian R. H. Raetz‡¶

From the ¶Department of Biochemistry, Duke University Medical Center, Box 3711, Durham, North Carolina 27710 and the §Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Two-thirds of the lipid A in wild-type Escherichia coli K12 is a hexa-acylated disaccharide of glucosamine in which monophosphate groups are attached at positions 1 and 4'. The remaining lipid A contains a monophosphate substituent at position 4' and a pyrophosphate moiety at position 1. The biosynthesis of the 1-pyrophosphate unit is unknown. Its presence is associated with lipid A translocation to the outer membrane (Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., and Raetz, C. R. H. (1998) J. Biol. Chem. 273, 12466–12475). To determine if a phosphatase regulates the amount of the lipid A 1-pyrophosphate, we grew cells in broth containing nonspecific phosphatase inhibitors. Na₂WO₄ and sodium fluoride increased the relative amount of the 1-pyrophosphate slightly. Remarkably, NH₄VO₃-treated cells generated almost no 1-pyrophosphate, but made six monophosphate slightly. Remarkably, NH₄VO₃-treated cells generated almost no 1-pyrophosphate, but made six monophosphate slightly. Remarkably, NH₄VO₃-treated cells generated almost no 1-pyrophosphate, but made six monophosphate slightly.

With the exception of the reaction that generates the 1-pyrophosphate unit (Fig. 1A), all the enzymes required for making lipid A in E. coli K12 are now known (1, 13, 14). However, in Salmonella typhimurium and Salmonella minnesota, additional lipid A species derivatized with palmitate, 4-amino-4-deoxy-L-arabinose (L-4-aminoarabinose), S-3-hydroxymyristoyl groups are further acylated with laurate and myristate, respectively (1, 2). A nonrepeating oligosaccharide known as the core is attached at the 6' position of lipid A (1–4). In the proper genetic setting, the outer core sugars may be further glycosylated with polymeric O-antigen (1–7).

Lipid A recovered from wild-type E. coli K12 (designated the “bis-phosphate”) contains monophosphate substituents at positions 1 and 4’ (Fig. 1A). The rest (termed the “lipid A 1-pyrophosphate”) contains a monophosphate group at 4’ and an unsubstituted pyrophosphate unit at 1 (Fig. 1A) (10–12). The 2’ and 3’ R-3-hydroxymyristoyl groups are further acylated with laurate and myristate, respectively (1, 2). A nonrepeating oligosaccharide known as the core is attached at the 6’ position of lipid A (1–4). In the proper genetic setting, the outer core sugars may be further glycosylated with polymeric O-antigen (1–7).

With the exception of the reaction that generates the 1-pyrophosphate unit (Fig. 1A), all the enzymes required for making lipid A in E. coli K12 are now known (1, 13, 14). However, in Salmonella typhimurium and Salmonella minnesota, additional lipid A species derivatized with palmitate, 4-amino-4-deoxy-L-arabinose (L-4-aminoarabinose), S-3-hydroxymyristoyl groups are further acylated with laurate and myristate, respectively (1, 2). A nonrepeating oligosaccharide known as the core is attached at the 6’ position of lipid A (1–4). In the proper genetic setting, the outer core sugars may be further glycosylated with polymeric O-antigen (1–7).

Lipopolysaccharide is a major component of the outer leaflet of the outer membranes of Gram-negative bacteria (1–7). The hydrophobic anchor of lipopolysaccharide, termed lipid A (1, 2, 8, 9), is a β(1’-6)-linked disaccharide of glucosamine. In Escherichia coli K12, the 2, 3, 2’, and 3’ positions of the disaccharide are acylated with R-3-hydroxymyristoyl groups, and the 1 and 4’ positions are phosphorylated (Fig. 1A). Two-thirds of the lipid A recovered from wild-type E. coli K12 (designated the “bis-phosphate”) contains monophosphate substituents at positions 1 and 4’ (Fig. 1A). The rest (termed the “lipid A 1-pyrophosphate”) contains a monophosphate group at 4’ and an unsubstituted pyrophosphate unit at 1 (Fig. 1A) (10–12). The 2’ and 3’ R-3-hydroxymyristoyl groups are further acylated with laurate and myristate, respectively (1, 2). A nonrepeating oligosaccharide known as the core is attached at the 6’ position of lipid A (1–4). In the proper genetic setting, the outer core sugars may be further glycosylated with polymeric O-antigen (1–7).

With the exception of the reaction that generates the 1-pyrophosphate unit (Fig. 1A), all the enzymes required for making lipid A in E. coli K12 are now known (1, 13, 14). However, in Salmonella typhimurium and Salmonella minnesota, additional lipid A species derivatized with palmitate, 4-amino-4-deoxy-L-arabinose (L-4-aminoarabinose), S-3-hydroxymyristoyl groups are further acylated with laurate and myristate, respectively (1, 2). A nonrepeating oligosaccharide known as the core is attached at the 6’ position of lipid A (1–4). In the proper genetic setting, the outer core sugars may be further glycosylated with polymeric O-antigen (1–7).

High levels of 1-4-aminoarabinose are made in polymyxin-resistant mutants of S. typhimurium, which harbor lesions in another two component regulatory system, known as PmrA/PmrB (24, 25). The latter is thought to be downstream of and activated by PhoP/PhoQ (16, 19). Polymyxin-resistant mutants of E. coli K12 (26, 27) have recently been characterized, and like strains of Salmonella, they synthesize significant amounts of lipid A species bearing palmitate, 1,4-aminoarabinose, and/or phosphoethanolamine (27). E. coli K12 must therefore possess the enzymatic machinery to generate these substitutions, despite their absence in cells grown on nutrient broth.

An operon of PhoP/PhoQ-regulated genes that is required for the maintenance of polymyxin resistance (and possibly for 1,4-aminoarabinose biosynthesis) has recently been discovered in both S. typhimurium and E. coli K12 (18). The regulatory and enzymatic functions of the products encoded by these genes have not yet been elucidated (18). A separate PhoP/PhoQ-
regulated gene \((\text{pagP})\), which is required for resistance to a subset of the antibacterial polypeptides present in neutrophils (17), may encode the enzyme that incorporates the palmitate residue found in some lipid A molecular species of \(S.\ typhimurium\). However, \(\text{pagP}\) is not part of the \(\text{l}-4\)-aminoarabinose gene cluster (17).

We now report that six major lipid A variants derivatized with palmitate, \(\text{l}-4\)-aminoarabinoside, and/or phosphoethanolamine (Fig. 1C) accumulate in wild-type cells of \(E.\ coli\ K12\) treated with \(25 \text{ mM NH}_4\text{VO}_3\), despite their complete absence under ordinary growth conditions (Fig. 1A). The lipid A modifications induced by \(\text{NH}_4\text{VO}_3\) in \(E.\ coli\ K12\) resemble those seen in untreated strains of \(S.\ typhimurium\) (15, 16, 18, 28, 29), but their induction in \(E.\ coli\) is not dependent upon a functional PhoP/PhoQ signaling system, suggesting that \(\text{NH}_4\text{VO}_3\) acts downstream of PhoP/PhoQ, perhaps on PmrA/PmrB. We have devised methods for isolating milligram quantities of several of these substances released from cells by pH 4.5 hydrolysis at 100 °C in SDS (10, 30, 35). To label lipid A with \(32\text{P}\), cells were grown and extracted, hydrolyzed before use. LB broth containing 25 mM \(\text{NH}_4\text{VO}_3\) was mixed equal volumes of autoclaved (2-fold concentrated) LB medium and filter-sterilized aqueous 50 mM \(\text{NH}_4\text{VO}_3\).

\textbf{Analysis of Lipid A Released from \(32\text{P}\)-Labeled Cells by Mild Acid Hydrolysis}—To label lipid A with \(32\text{P}\), cells were grown and extracted, hydrolyzed before use. LB broth containing 25 mM \(\text{NH}_4\text{VO}_3\) was mixed equal volumes of autoclaved (2-fold concentrated) LB medium and filter-sterilized aqueous 50 mM \(\text{NH}_4\text{VO}_3\).

\textbf{Analysis of Lipid A Released from \(32\text{P}\)-Labeled Cells by Mild Acid Hydrolysis}—To label lipid A with \(32\text{P}\), cells were grown and extracted, hydrolyzed before use. LB broth containing 25 mM \(\text{NH}_4\text{VO}_3\) was mixed equal volumes of autoclaved (2-fold concentrated) LB medium and filter-sterilized aqueous 50 mM \(\text{NH}_4\text{VO}_3\).

\textbf{Analysis of Lipid A Released from \(32\text{P}\)-Labeled Cells by Mild Acid Hydrolysis}—To label lipid A with \(32\text{P}\), cells were grown and extracted, hydrolyzed before use. LB broth containing 25 mM \(\text{NH}_4\text{VO}_3\) was mixed equal volumes of autoclaved (2-fold concentrated) LB medium and filter-sterilized aqueous 50 mM \(\text{NH}_4\text{VO}_3\).
were separated by low speed centrifugation, and the lower and the upper phases were collected. The upper phase was extracted with 80 ml of lower phase derived from a fresh neutral two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:3:1, v/v). The lower phase was extracted with 80 ml of the upper phase from the same fresh neutral two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:3:1, v/v). One-fourth of the total dried lipid A sample described above was re-dissolved in 10 ml of chloroform/methanol/water (2:3:1, v/v). The material was centrifuged at low speed to remove insoluble debris, and the supernatant was loaded onto the column at its natural flow rate. The column then was washed with 12 ml of chloroform/methanol/water (2:3:1, v/v), Fractions (2 ml each) were collected. Lipids EV5 and EV6 were eluted with 12 ml of chloroform/methanol/80 mM aqueous ammonium acetate (2:3:1, v/v). Lipids EV2, EV3, and EV4 were eluted with 12 ml of chloroform, methanol, 120 mM ammonium acetate (2:3:1, v/v). The "normal" hexa-acylated lipid A 1,4'-bis-phosphate and the hepta-acylated species EV1 were eluted with 12 ml of chloroform/methanol/240 mM ammonium acetate (2:3:1, v/v). Finally, the lipid A was eluted with 12 ml of chloroform/methanol/methanol/80 mM aqueous ammonium acetate (2:3:1, v/v). The “normal” hexa-acylated lipid A 1,4'-bis-phosphate from untreated cells) were further purified by preparative thin layer chromatography. Lipid A samples from the dried DEAE cellulose column fractions were re-dissolved in chloroform/methanol (4:1, v/v), and each ~0.5-mg sample was applied in a line to a 20 × 20-cm silica Gel 60 TLC plate (0.25 mm thickness). The plates were developed in the solvent chloroform/pyridine/88% formic acid, water (50:50:16.5, v/v). The spots were visualized by charring on a hot plate after spraying the chromatogram with a mixture of ethanol/bis-anisaldehyde/H2SO4/acetic acid (89:2.5:4:1, v/v) (36). The DEAE cellulose fractions containing the lipids of interest were then converted to neutral two-phase Bligh/Dyer mixtures by addition of the necessary amounts of chloroform and water. The lower phases were pooled, as appropriate, dried under N2, and stored at −20 °C.

With the exception of EV5, which was produced in much lower quantities, the substituted lipid A derivative EV1, EV2, EV3, EV4, and EV6 that accumulated in NH4VO3-treated cells (as well as the lipid A 1,4'-bis-phosphate from untreated cells) were further purified by preparative thin layer chromatography. Lipid A samples from the dried DEAE cellulose column fractions were re-dissolved in chloroform/methanol (4:1, v/v), and each ~0.5-mg sample was applied in a line to a 20 × 20-cm silica Gel 60 TLC plate (0.25 mm thickness). The plates were developed in the solvent chloroform/pyridine/88% formic acid, water (50:50:16.5, v/v). While the plates were drying at room temperature, the lipid A bands could be seen transiently as white zones. These were removed with a pencil, and the plates were then dried to complete at room temperature for ~20 min before each marked zone was scraped off the plate with a clean razor blade. Each lipid A derivative was extracted from the silica chips with 24.0 ml of an acidic single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/240 mM aqueous ammonium acetate adjusted to pH 1.5 with HCl (1:2:0.8, v/v). The silica chips were removed by low speed centrifugation, and the supernatant was collected and passed through a layer of glass wool stuffed into a Pasteur pipette in order to remove any residual silica. The filtered material was converted to a two-phase Bligh/Dyer mixture by addition of 6.0 ml each of chloroform and water. The lower phase was collected and neutralized by the addition of 24 drops of pyridine prior to the addition of 30 drops of extra methanol to clear the solution. The lower phase was then dried under a stream of N2. Finally, prior to mass spectrometry, the TLC-purified lipid A derivatives were subjected to a second DEAE cellulose column chromatography, as described above, to remove contaminating metal ions. The purified lipid A derivatives were stored at −20 °C prior to mass spectrometry and between purification steps.

Mass Spectrometry Analysis of Purified Lipid A Derivatives—Spectra were acquired in the negative linear mode by using a time of flight matrix-assisted laser desorption/ionization mass spectrometer (Kratos Analytical Manchester, United Kingdom), equipped with a 237-nm nitrogen laser and set at a 20-kV extraction voltage (37). Each spectrum was the average of 20 shots. Two kinds of matrices were used in the present study. One was a saturated solution of 2,5-dihydroxybenzoic acid in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) for EV1, EV4, and EV5. Lipid samples were dissolved in a mixture of chloroform/methanol (4:1, v/v) before mixed with a matrix (1:1, v/v) on a slide. The sample mixtures were allowed to dry at room temperature prior to mass analysis.

Mass Spectrometry of the Aminodeoxypentose Released from Purified Lipid A—The spectrum was acquired in positive mode on a JEOL JMS-SX-102 high resolution mass spectrometer (Physical Science Laboratory, Department of Chemistry, Duke University) at 62.5 °C with a fast atom bombardment (FAB) gun set at 8 kV and a 10 kV acceleration voltage.

Results

Lipid A 1-Pyrophosphate Levels in E. coli Mutants Lacking the Phosphatidylglycerophosphate Phosphatases—In previous studies (12), the molar ratio of the hexa-acylated lipid A 1,4'-bis-phosphate to the lipid A 1-pyrophosphate (Fig. 1A) was shown to be about 2.5 in wild-type strains of E. coli grown on nutrient broth. Mass spectrometry confirmed that the pyrophosphate unit was indeed attached to position 1 of the glucosamine disaccharide (Fig. 1A) (12). To exclude the possibility that the two known phosphatidylglycerophosphate phosphatases of E. coli (PgpA and PgpB) (38) are involved in regulating the levels of the lipid A 1-pyrophosphate, 32P-labeled lipid A species from the phosphatase-defective strains CF10 (ppgA-) CT20 (ppgB+), and CF30 (ppgA+ ppgB-) (39) were prepared and analyzed. The ratio of lipid A 1,4'-bis-phosphate to lipid A 1-pyrophosphate was not altered in these mutants when compared with wild-type (data not shown).

Covalent Modifications of Lipid A in E. coli Cells Treated with NH4VO3—To examine the possibility that other (as yet uncharacterized) phosphatases might play a role in determining the amount of the lipid A 1-pyrophosphate, E. coli cells were treated with Na2WO4, NH4VO3, and sodium fluoride. These compounds are nonspecific phosphatase inhibitors that have been used to perturb the levels of lipid intermediates in some systems (40). An overnight culture of E. coli W3110 was grown on LB broth at 37 °C, and was diluted 100-fold into separate culture tubes each of which contained 5 ml of fresh LB medium supplemented with 5, 10, 25, or 50 mM Na2WO4, NH4VO3, or sodium fluoride. Next, 5 μCl/ml 32P-Pi was added to each diluted culture. Cells were grown at 42 °C for 3 h. Lipid A from the cells in each 32P-labeled culture was released by pH 4.5 hydrolysis (12) and analyzed by thin layer chromatography (Fig. 2). In the Na2WO4 and the sodium fluoride-treated cells, the lipid A profiles were similar to that of untreated cells (Figs. 2 and 3) with only slightly elevated relative levels of the 1-pyrophosphate species.

Unexpectedly, the lipid A 1-pyrophosphate disappeared altogether in cells treated with 25 mM NH4VO3 (Figs. 2 and 3). However, at least six new major lipid species were observed, which generally migrated more slowly than the predominant hexa-acylated lipid A 1,4'-bis-phosphate found in the untreated cells. Based on their mobility on TLC plates, they were designated EV1 to EV6 (Figs. 2 and 3). In control experiments, in which 25 mM NH4Cl was included in the LB broth instead of 25 mM NH4VO3, no lipid A modifications were observed (not shown), indicating that the VO3− anion and/or its oligomers were responsible for the effect. In the presence of 25 mM NH4VO3, the cells grew slower, but their doubling time was only lengthened by ~20% (data not shown).

To obtain preliminary evidence that the NH4VO3-induced compounds are indeed lipid A derivatives, several of them (EV3, 4, and 5/6) were isolated from a culture of 32P-labeled E. coli W3110 grown on LB broth containing 25 mM NH4VO3 by pH 4.5 hydrolysis and TLC. Each compound was then further hydrolyzed in 0.2 M HCl at 100 °C for 90 min to convert any
lipid A species that might be present to its 4′-monophosphate derivative (32, 33). The unknown substances EV3, 4, and 5/6 all yielded the same pattern of lipid A 4′-monophosphates that were obtained by 0.2 M HCl hydrolysis of the hexa-acylated lipid A 1,4′-bis-phosphate obtained from wild-type E. coli (data not shown). Accordingly, the above compounds from the NH₄VO₃-treated cells appear to be a family of related lipid A derivatives substituted with acid labile hydrophilic groups. However, compound EV1 is likely to be a hepta-acylated lipid A 1,4′-bis-phosphate (Fig. 1C), based on its TLC migration (Figs. 2 and 3) and physical characterization (see below), and EV2 also contains a hepta-acylated lipid A moiety (see below). The relative amounts of EV1 to EV6 varied slightly depending upon the growth conditions, the strain, and the protocol for 32P labeling (Figs. 2 and 3).

Lipid A Modifications in E. coli Cells Treated with NH₄VO₃ Resemble Those Normally Found in Salmonella—To test if NH₄VO₃ had any effect on lipid A biosynthesis in S. typhimurium, wild-type cells of strain LT2 were labeled and grown on LB broth in the presence or absence of 25 mM NH₄VO₃. Lipid A species were then analyzed in parallel with lipid A from NH₄VO₃-treated E. coli W3110 (Fig. 3). Even in the absence of NH₄VO₃, S. typhimurium LT2 produced a complex series of lipid A derivatives, some of which migrated like the species observed in NH₄VO₃-treated E. coli (Fig. 3). There was very little lipid A 1-pyrophosphate in S. typhimurium LT2 cells under any condition. This TLC analysis suggests that NH₄VO₃ induces E. coli K12 to synthesize lipid A modifications that are
Well as the unsubstituted hexa-acylated lipid A 1,4-bis-phosphate. Finally, EV1, the hexa-acylated lipid A 1,4-bis-phosphate controls the negative-ion mode. The hexa-acylated lipid A 1,4-bis-phosphate from E. coli K12 W3110 served as the control. The spectrum of the latter (Fig. 5) was characterized by a prominent peak at m/z 1796.8, consistent with [M − H]− for the structure shown in Fig. 1A (Mr = 1798.4) and previous reports (11, 15, 35, 41). The small peak at m/z 1818.1 (Fig. 5) in the spectrum of the hexa-acylated lipid A 1,4-bis-phosphate was interpreted as [M + Na−2H]−.

The negative-ion spectrum of EV1 (Fig. 5) demonstrated a major peak at m/z 2035.4, consistent with the molecular ion [M − H]− of a hepta-acylated lipid A 1,4-bis-phosphate species bearing a palmitoyl group, as shown in Fig. 1C (Mr = 2036.8). The negative-ion spectrum of EV2 (Fig. 5) showed a major peak at m/z 2166.8, consistent with [M − H]− of the hepta-acylated lipid A bis-phosphate species EV1, bearing an additional amidoxypentose substituent (Fig. 1C, Mr = 2167.9). The smaller peak at m/z 2036.3 (Fig. 5) in EV2 was attributed to loss of the amidoxypentose moiety (16, 28, 42), which is attached via a rather labile phosphodiester linkage. This fragmentation presumably occurred during mass spectrometry, as the sample migrated like a single pure compound during TLC.

The negative-ion spectrum of EV3 (Fig. 5) revealed a prominent molecular ion [M − H]− at m/z 1928.3, corresponding to a hexa-acylated lipid A bis-phosphate species derivatized with one amidoxypentose moiety, as shown in Fig. 1C (predicted Mr = 1929.5). The smaller peaks at m/z 1797.1 and m/z 1702.1 were attributed to the loss of the amidoxypentose moiety or of an R-3-hydroxymyristoyl residue, respectively. The extent of fragmentation during mass analysis varied slightly from sample to sample.

The negative-ion spectrum of EV4 (Fig. 5) demonstrated a major peak at m/z 1919.3, consistent with a molecular ion [M − H]− of hexa-acylated lipid A bis-phosphate substituted with an extra phosphoethanolamine moiety, as in Fig. 1C (Mr = 1921.4). The negative-ion spectrum of EV6 (Fig. 5) showed a molecular ion at m/z 2051.7, interpreted as [M − H]− of a hepta-acylated lipid A bis-phosphate substituted with both an amidoxypentose residue and a phosphoethanolamine moiety, as shown in Fig. 1C (Mr = 2052.6). Loss of the amidoxypentose residue would account for the peak at m/z 1921.4.

EV5 was recovered together with another minor co-migrating lipid, which was not removed because of the low abundance of EV5 (Figs. 3 and 4). The negative-ion spectrum of EV5 (Fig. 5) revealed at least two molecular ions. The minor one at m/z 2289.1 was consistent with the [M − H]− of the hepta-acylated lipid A bis-phosphate EV1, further substituted with one amidoxypentose residue and one phosphoethanolamine substituent, as shown in Fig. 1C (Mr = 2291.0). The more prominent peak at m/z 2043.1 was interpreted as the [M − H]− of a hexa-acylated lipid A bis-phosphate further substituted with two phosphoethanolamine moieties (Mr = 2044.5) (Fig. 1C and

---

2Z. Zhou, A. A. Ribeiro, and C. R. H. Raetz, manuscript in preparation.
other modifications of lipid A are usually seen in S. typhi-

m M, lane 3 methanol/water (2:3:1, v/v) (species at

arise by loss of one phosphoethanolamine residue from the

ions (41), B1 positive-ion spectrum of EV4 (not shown) revealed two oxonium

both the positive- and negative-ion modes of mass analysis. The

under the conditions employed. On the other hand, the phos-

stitution, since the relevant linkages appear to be too labile

any information regarding the site of aminodeoxypentose sub-

assigned.

Lipid A species released from 1 liter of cells by hydrolysis at pH 4.5 were dissolved in 10 ml of chloroform/methanol/water (2:3:1, v/v). The

column.

sample was loaded onto a 2-ml column of DEAE cellulose (Whatman DE52, acetate form) in the same solvent. A 20-

TLC plate. The plate was developed in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16:5, v/v). The lipids on the plates were

visualized by sulfuric acid charring. The fact that both the B 1

substituent in EV4 is likely to be attached at the 1-position

not substituted in EV4. Therefore, the phosphoethanolamine

bis

(Fig. 1

Fig. 5 all appear to contain three kinds of substituents singly or

the molecular ion at

m/z

2043.1. The peak at

m/z

an aminodeoxypentose residue from the species at

m/z

2043.1. The peak at

m/z

1822.3 could not be

assigned.

MALDI/TOF analysis was also conducted in the positive-ion mode (not shown) in an attempt to determine the sites of attachment of the aminooxypentose and the phosphoethanol-
amine residues. Although the positive-ion spectra were entirely consistent with the results shown in Fig. 5, they did not provide

any information regarding the site of aminooxypentosyl substitu-
tion, since the relevant linkages appear to be too labile under the conditions employed. On the other hand, the phos-

phosphoethanolamine substitutions in EV4 and EV6 were stable in both the positive- and negative-ion modes of mass analysis. The positive-ion spectrum of EV4 (not shown) revealed two oxonium ions (41), B1 at m/z 1088.1 and B2 at m/z 1702.2, as well as the molecular ion at m/z 1921.2, corresponding to [M + H]+. The fact that both the B1 and B2 of EV4 were the same as those observed for the unmodified hexa-acylated lipid A 1,4-

bis-phosphate (not shown) indicated that the 4′-phosphate was not substituted in EV4. Therefore, the phosphoethanolamine

substituent in EV4 is likely to be attached at the 1-position (Fig. 1C).

In summary, the substituted lipid A derivatives analyzed in

Fig. 5 all appear to contain three kinds of substituents singly or

in combination: a palmitoyl group, an aminooxypentosyl residue, and one (or two) phosphoethanolamine moieties (Fig. 1C

and Table II). The combinations of these substituents account

for the micro-heterogeneity of the lipid A species associated

with NH4VO3 treatment of E. coli K12. Interestingly, these and other modifications of lipid A are usually seen in S. typhi-
murium cells grown on nutrient broth without any special

treatments (Figs. 1B and 3) (16, 18, 28, 29). In E. coli K12, they

have been reported only in polymyxin-resistant mutants (27).

FAB Mass Spectrometry of the Aminooxypentose Substitu-

tent—Although stable for days at 25 °C during DEAE cellulose

chromatography (Fig. 4) in chloroform/methanol/water (2:3:1, v/v), all samples, including the lipid A 1,4′-bis-phosphate, EV2, EV3, and EV6, decomposed within hours when dissolved in CDCl3/CD3OD (4:1, v/v). As shown in Fig. 6, the resulting degradation products included several rapidly migrating, par-
tially deacylated lipid A 4′-monophosphates (Fig. 6, lanes 1–4), all of which had lost their 1-phosphate substituents dur-
ing exposure to CDCl3/CD3OD (4:1, v/v). The three metavanadate-

induced lipid A derivatives (EV2, 3, and 6) generated an addi-
tional, slowly migrating substance (Fig. 6, lanes 2–4), not seen in the degradation products of the hexa-acylated lipid A 1,4′-

bis-phosphate (Fig. 6, lane 1). This hydrophilic compound, which is readily detected by charring with sulfuric acid, is the

aminooxypentosyl moiety of EV2, 3, and 6 (see below). To isolate this material, the lipid A samples that had been exposed to CDCl3/CD3OD (4:1, v/v) for 3 days at room temperature were dried under N2 and were resuspended in a neutral, two-phase

Bligh/Dyer system, consisting of chloroform/methanol/water (2:1:8, v/v). The rapidly migrating degradation products partitioned into the lower phase, and the slowly migrating material

was recovered in the upper phase (Fig. 6, lanes 5–7). The upper

phase of each sample was washed twice with fresh pre-equili-

brated lower phase to remove residual lipids, and the upper

phases were then dried. The hydrophilic substances released in this way from EV2, 3, and 6 all stained with ninhydrin (not shown), confirming the presence of an amino group.

The positive-ion FAB mass spectrum of the hydrophilic material released from EV2 showed a prominent molecular ion [M + H]+ at m/z 150.11 (Fig. 7). This is consistent with the

Table II). The peak at m/z 2158.8 was attributed to the loss of an aminooxypentosyl residue from the species at m/z 2289.1.

The peak at 2064.9 was attributed to a sodium adduct of the

at

m/z

2043.1. The peak at

m/z

1921.2, corresponding to [M

1

- phosphate (not shown) indicated that the 4

9

- monophosphates (Fig. 6, lanes 1–4) (16, 18, 28, 29). In E. coli K12, they

have been reported only in polymyxin-resistant mutants (27).

FIG. 4. Separation and large scale preparation of lipid A derivatives that accumulate in NH4VO3-treated cells on a DEAE cellulose column. Lipid A species released from 1 liter of cells by hydrolysis at pH 4.5 were dissolved in 10 ml of chloroform/methanol/water (2:3:1, v/v). The sample was loaded onto a 2-ml column of DEAE cellulose (Whatman DE52, acetate form) in the same solvent. A 20-μl portion of the initial sample was spotted (Load, lane 1). The run-through was collected as a single fraction (RT, lane 2). The column was washed with 12 ml of chloroform/ methanol/water (2:3:1, v/v) (0 mm, lane 3), collected as a single fraction. The column was then eluted with 12 ml of chloroform/methanol/60 mM ammonium acetate in water (2:3:1, v/v) (lane 4), 12 ml of chloroform/methanol/120 mM ammonium acetate (2:3:1, v/v) (lanes 5–10), 12 ml of chloroform/methanol/240 mM ammonium acetate (2:3:1, v/v) (only the first 4 fractions out of six are shown, lanes 11–14), and 12 ml of chloroform/methanol/480 mM ammonium acetate (2:3:1, v/v) (fractions not shown). A 20-μl sample from each fraction was spotted onto a 10 × 20-cm Silica Gel 60 TLC plate. The plate was developed in the solvent of chloroform/pyridine/88% formic acid, water (50:50:16.5, v/v). The lipids on the plates were visualized by sulfuric acid charring.
elemental composition of an aminodeoxypentose, like 4-amino-4-deoxy-L-arabinose, the molecular weight of which is 149.15 (42).

Analysis of the Aminodeoxypentose Substituent Released from EV3 by 1H NMR—As shown in Fig. 8 and Table III, 1H NMR experiments were used to characterize the structure of the putative 4-amino-4-deoxy-L-arabinose released from EV3. Since the anomeric OH of the released sugar is no longer phosphorylated after exposure to CDCl3/CD3OD (4:1, v/v), two anomeric forms (designated A and B in Fig. 8) were detected in the spectrum. Based upon the proton connectivities determined by two-dimensional COSY experiments (not shown), the indi-
MALDI/TOF mass analysis of lipid A species purified from E. coli K12 grown on LB broth in the presence of 25 mM NH₄VO₃

| Lipid A species | [M – H] | Mr | Modification |
|-----------------|--------|----|--------------|
| 1,4'-bis-Phosphate | 1796.8 | 1798.4 | C₁₆₀ L-Ara₄N pEtN |
| EV1 | 2035.4 | 2036.8 | 0 0 0 |
| EV2 | 2186.8 | 2187.9 | 1 0 0 |
| EV3 | 1928.3 | 1932.5 | 1 0 0 |
| EV4 | 1928.3 | 1929.5 | 0 0 1 |
| EV5 | 2043.1 | 2044.5 | 0 0 2 |
| EV6 | 2051.7 | 2052.6 | 0 1 1 |

The Mr is the calculated molecular weight for the proposed structure (see also Fig. 1C). [M – H]⁺ is the observed molecular ion determined in the experiment shown in Fig. 5.

FIG. 7. Positive mode FAB mass spectrum of the aminodeoxypentose released from EV2. The upper phase of EV2, prepared as described in the legend to Fig. 6, was collected and extracted twice with several milliliters of a lower phase from a fresh two phase Bligh/Dyer system. The washed upper phase was dried by lyophilization, and the water-soluble compound released from EV2 was analyzed by FAB mass spectrometry. The spectrum was the average of 7 scans.

FIG. 8. 500 MHz ¹H NMR spectrum of the aminodeoxypentose released from EV3. The aminodeoxypentose, as isolated by the protocols described in the legends to Figs. 6 and 7, was dried and redissolved in 0.6 ml of D₂O. The 500 MHz ¹H NMR spectrum shown was recorded at 25 °C with the HDO signal referenced to 4.80 ppm. Sugar resonances were assigned by two-dimensional COSY analysis (not shown). The sugar resonances corresponding to the two anomeric forms of the aminodeoxypentose released from EV3 are designated A and B. The equatorial and axial protons at the 5 position are designated H-5e and H-5a.

individual proton resonances of the two anomeric species could be assigned. Their chemical shifts (ppm) and vicinal coupling constants (JH-H2) were measured directly from the one-dimensional ¹H NMR spectrum (Table III). The chemical shifts were referenced to the internal HDO signal at 4.80 ppm and were compared with the data previously reported for chemically synthesized 4-amino-4-deoxy-L-arabinose (42) (Table III). The shapes of the individual proton resonances in the spectrum of the A- and B-forms derived from EV3 and the vicinal coupling constants (JH-H2) (Fig. 8 and Table III) are indeed very similar to those of the α- and β-anomers of the standard (42). The only exceptions are the chemical shifts of the H-1 signals, which differ from the standard by 0.2–0.3 ppm. However, differences in the solvent acidity of the EV-3-derived sample and the previously reported standard (42) might account for these minor discrepancies, since the pH was not carefully controlled. Taken together with the previous work (23, 28, 29, 42) on 4-amino-4-deoxy-L-arabinose-modified lipid A species in Salmonella, it seems very likely that the aminodeoxypentose substituent present in the E. coli lipid A derivatives EV2, 3, and 6 is 4-amino-4-deoxy-L-arabinose. Detailed side by side comparisons of the ¹H NMR spectra of intact purified EV3 versus lipid IIₐ (a precursor isolated from Kdo-deficient mutants of S. typhimurium that is known to contain 4-amino-4-deoxy-L-arabinose) (28, 29, 42) further confirm the above assignments.³

NH₄VO₃ Induces Lipid A Substitutions in phoQ and phoP/Q Deletion Mutants—To determine if NH₄VO₃ induction of lipid A modifications in E. coli requires the PhoP/PhoQ system, as is the case for S. typhimurium grown in the absence of NH₄VO₃ (16, 18), the E. coli phoQ and phoP deletion mutants CSH26ΔQ and CSH26ΔPQ (43) were grown in the presence of 25 mM NH₄VO₃ and ³²P. The lipid A species were then released from the cells by pH 4.5 hydrolysis, and were analyzed by thin layer chromatography and PhosphorImager analysis (Fig. 9). Untreated cells of all strains contained the usual hexa-acylated lipid A 1,4'-bis-phosphate and the lipid A 1-pyrophosphate. NH₄VO₃-treated CSH26ΔQ and CSH26ΔPQ cells generated a

³ C. Waldburger, unpublished data.
coli lipid A (33, 44). Accordingly, the wild-type and mutant cells of E. coli clearly demonstrate that NH\textsubscript{4}VO\textsubscript{3} induction does not require the PhoP/PhoQ system, perhaps because NH\textsubscript{4}VO\textsubscript{3} acts downstream of the PhoP/PhoQ system, a global regulatory network that controls over 40 genes and is essential for pathogenesis (16, 19). In the case of the lipid A modifications, the PhoP/PhoQ system generally functions by activating PmrA/PmrB, a separate two-component system that may directly activate the transcription of the genes encoding some of the relevant enzymes (18).

In the present study, we have discovered that 25 mM NH\textsubscript{4}VO\textsubscript{3} induces three kinds of covalent modifications of E. coli K12 lipid A, which resemble those normally found in S. typhimurium (Fig. 1, lanes B versus C), resulting in the accumulation of six major species (Figs. 1–5). Of these, EV1, 2, 3, 4, and 6 have been purified to apparent homogeneity as judged by mass spectrometry and PhosphorImager analysis (Fig. 10). When grown on LB broth in the absence of NH\textsubscript{4}VO\textsubscript{3}, these three strains produced mainly hexa-, penta-, or tetra-acylated lipid A moieties, consistent with their genotypes (Fig. 10, lanes 1, 3, and 5). In each mutant, the expected 4’-monophosphate, 1,4’-bis-phosphate, and 1- pyrophosphate variants were also present. When treated with NH\textsubscript{4}VO\textsubscript{3}, all three strains generated a more complex series of slowly migrating lipid A derivatives at the expense of the 1-pyrophosphate species (Fig. 10, lanes 2, 4, and 6), consistent with the modifications shown in Fig. 1C. These findings indicate that the acyloxyacyl moieties of lipid A are not needed for the proper functioning of the enzymes that attach the NH\textsubscript{4}VO\textsubscript{3}-induced modifications.

**DISCUSSION**

The enzymes that generate the hexa-acylated lipid A 1,4’-bis-phosphate (Fig. 1A) found in E. coli K12 and other Gram-negative bacteria are well characterized (1, 13). However, many additional covalent modifications of lipid A have been reported. In S. typhimurium, for instance, lipid A derivatives exist that are modified with 4-amino-4-deoxy-l-arabinose, phosphoethanolamine, palmitate, and/or S-2-hydroxymyristate (Fig. 1B) (15, 28, 46). Structural diversity and partial substitution (Fig. 1B) give rise to a large number of distinct molecular species. While the existence of such lipid A modifications has been recognized for a long time (47, 48), the enzymes that generate them are still largely unknown.

Although not required for the growth under laboratory conditions, the modified lipid A species of S. typhimurium (Figs. 1B and 3) are interesting from the perspective of pathogenesis (16–18). Extensive modification of lipid A with 1,4-aminoarabinose is associated with resistance to polymyxin and other cationic antibacterial peptides (16–19, 24, 25). In S. typhimurium, formation of modified lipid A derivatives is under the control of the PhoP/PhoQ system, a global regulatory network that controls over 40 genes and is essential for pathogenesis (16, 19). In the case of the lipid A modifications, the PhoP/PhoQ system generally functions by activating PmrA/PmrB, a separate two-component system that may directly activate the transcription of the genes encoding some of the relevant enzymes (18).

**Table III**

| A-species<sup>a</sup> | H-1 (J<sub>1,2</sub>) | H-2 (J<sub>2,3</sub>) | H-3 (J<sub>3,4</sub>) | H-4 (J<sub>4,5</sub>) | H-5e (J<sub>5e,5a</sub>) | H-5a |
|----------------------|---------------------|---------------------|---------------------|---------------------|---------------------|------|
| from EV3             | 4.383               | 3.545               | 3.985               | 3.616               | 4.065               | 3.772 |
| α-anomer<sup>b</sup>| (7.1)               | (8.9)               | (4.7)               |                     |                     |      |
| (l-Ara4N)            | 4.57                | 3.41                | 3.94                | 3.6                 | 4.02                | 3.80  |
| B-species<sup>c</sup> | 4.890               | 3.786               | 4.107               | 3.658               | 4.065               | 3.850 |
| from EV3             | (3.3)               | (10.0)              | (4.7)               |                     |                     |      |
| β-Anomer<sup>b</sup>| 5.21                | 3.69                | 4.10                | 3.6                 | 4.16                | 3.71  |
| (l-Ara4N)            | (3.3)               | (9.1)               | (4.6)               |                     |                     |      |

<sup>a</sup>1H NMR spectra for the isolated aminodeoxypentose isomers released from EV3 were recorded at 500 MHz with the HDO signal referenced to 4.80 ppm, as described by Naleway et al. (42). The 500 MHz spectra were obtained with a digital resolution of 0.3 Hz/point. At the actual digital resolution, chemical shifts are accurate to ~0.001 ppm, and coupling constants (J) have an uncertainty of 0.3 Hz.

<sup>b</sup>1H NMR spectra for the synthetic l-Ara4N anomers were recorded at 270 MHz with the HDO signal referenced to 4.80 ppm by Naleway et al. (42).

**Fig. 9. Lipid A modifications are induced by NH\textsubscript{4}VO\textsubscript{3} in phoP and phoQ deletion mutants of E. coli.** Strains were grown at 37°C, labeled and analyzed, as described in the legend to Fig. 2, in the presence or absence of 25 mM NH\textsubscript{4}VO\textsubscript{3}.

Family transporter required for lipopolysaccharide export (12, 45). Lipid A species were released from the 32P-labeled cells by pH 4.5 hydrolysis, and were analyzed by thin layer chromatography and PhosphorImager analysis (Fig. 10). When grown on LB broth in the absence of NH\textsubscript{4}VO\textsubscript{3}, the PhoP/PhoQ system is suppressed (42). The 500 MHz spectra were obtained with a digital resolution of 0.3 Hz/point. At the actual digital resolution, chemical shifts are accurate to ~0.001 ppm, and coupling constants (J) have an uncertainty of 0.3 Hz.
Legend to Fig. 2, in the presence or absence of 25 mM NH4VO3. Strains were grown at 42 °C, labeled, and analyzed, as described in the legend to Fig. 2, in the presence or absence of 25 mM NH4VO3. Lanes 1 and 2, W3110 (wild-type); lanes 3 and 4, MLK1067 (msbB B); lanes 5 and 6, MLK986 (htrB B msbB B)pKW2 (msbA B).

Fig. 10. Lipid A modifications are induced by NH4VO3 in htrB and msbB mutants of E. coli lacking acyloxyacyl moieties. Strains were grown at 42 °C, labeled, and analyzed, as described in the legend to Fig. 2, in the presence or absence of 25 mM NH4VO3. Lanes 1 and 2, W3110 (wild-type); lanes 3 and 4, MLK1067 (msbB B); lanes 5 and 6, MLK986 (htrB B msbB B)pKW2 (msbA B).

described prior to the present investigation, as all previous structural investigations of lipid A modifications have been based on the use of mixtures (25, 27). EV2, 3, and 6 all contain an aminodeoxypentose group, very likely to be 4-amino-4-deoxy-L-arabinose, as judged by NMR spectroscopy (Fig. 8 and Table II) of the substituent released from EV3. EV1 and EV2 (Fig. 1C and Table II) are characterized by the presence of hepta-acylated lipid A moieties. EV4, EV5, and EV6 (Fig. 1C and Table II) contain phosophothanolamine substituents. Although the NH4VO3 effect does not require a functional PhoP/PhoQ system (Fig. 9), it may be that metavanadate (or one of its oligomers) activates PmrA/PmrB by blocking the action of a key regulatory phosphatase (49). Interestingly, NH4VO3 has no effect on the composition of lipid A in pmrA-deficient mutants of S. typhimurium. Whatever its mechanism, the NH4VO3 effect opens the possibility of investigating the enzymology of lipid A modifications in diverse strains of E. coli K12, the organism in which most studies of lipid A biosynthesis have been conducted (1, 8, 9). In this context, it is already clear that NH4VO3-induced lipid A modifications do not require the presence of the acyloxyacyl groups (Fig. 10).

Mass spectrometry of the lipid A derivatives isolated from NH4VO3-treated E. coli failed to show the presence of S2-hydroxymyristate, which is easily detected in S. typhimurium lipid A under conditions of PhoP/PhoQ activation (16). It may be that E. coli can generate only a subset of the lipid A modifications that are found in S. typhimurium, suggesting the existence of additional biosynthetic enzymes in the latter organism. Alternatively, NH4VO3 treatment of E. coli may not activate the entire enzymatic system that is involved in lipid A modification.

It has not yet been demonstrated unequivocally that the 4-amino-4-deoxy-L-arabinose moiety is always attached to the 4'-phosphate and that the phosphoethanolamine residue is predominantly found at the 1-phosphate of lipid A in NH4VO3-treated E. coli, as suggested in Fig. 1C. In the lipid A precursors that accumulate in Kdo-deficient mutants of S. typhimurium, the 4-amino-4-deoxy-L-arabinose is attached to the 1-phosphate and the phosophothanolamine is on the 4'-phosphate (28, 29). Further characterization of EV2, 3, 4, and 6 by 1H and 31P NMR spectroscopy is in progress and should establish the sites at which these modifications are attached. The locations of the 4-amino-4-deoxy-L-arabinose and phosphothanolamine substitutions also need to be reinvestigated in the mature lipid A of wild-type S. typhimurium (Figs. 1B and 3). Purification of homogeneous molecular species based on the new procedures described above should greatly facilitate this effort.

It has been suggested that the 4-amino-4-deoxy-L-arabinose-substituted lipid A species seen in polymyxin-resistant mutants of S. typhimurium and E. coli reduce the overall negative charge of the lipopolysaccharide, thereby reducing the binding of polycationic antibiotics (50, 51). An attempt to show that NH4VO3-treated cells are polymyxin-resistant was unsuccessful, because polymyxin precipitated in the presence of 5 mM NH4VO3.

The enzymes that catalyze the lipid A modifications shown in Fig. 1, B and C, remain to be characterized. The ethanolamine phosphate groups found in EV4, EV5, and EV6 might be derived from phosphatidylethanolamine (52), but so far, no in vitro systems have been developed. A membrane-bound palmitoyltransferase that uses glycerophospholipids as the palmitate donor was previously shown to convert the diacylated myristyltransferase (53). This unusual acyltransferase has recently been shown to incorporate the palmitate moiety in EV1 and S. typhimurium lipid A (Fig. 1, B and C) (54). Genetic and enzymatic studies have revealed that the pagP gene (17), which is present in both S. typhimurium and E. coli, encodes the palmitoyltransferase (54).

Although the enzymes that generate L-4-aminoarabinose are obscure, a hypothetical pathway can now be proposed (Fig. 11). The important studies of Gunn et al. (18) have recently revealed the existence of several genes in S. typhimurium and E. coli required for the maintenance of polymyxin resistance. For instance, mutations in the ugd/pmrE or in the pmrF genes render S. typhimurium polymyxin-sensitive and incapable of making aminoarabinose under conditions of PhoP/PhoQ activation (18). We therefore suggest (Fig. 11) that the UDP-glucose dehydrogenase (Ugd/PmrE) could initiate the L-4-aminoarabinose pathway, in analogy to the role of this enzyme in the biosynthesis of UDP-xyllose in plants (55, 56). Orf3, which is encoded by one of the genes of unknown function found in the pmrF cluster (18), might then catalyze the oxidation of the 4-position (Fig. 11). Orf3 shows a high degree of similarity to enzymes that oxidize the 4-OH of pyranoses, such as UDP-galactose 4-epimerase. Decarboxylation of the intermediate generated by Orf3 might be spontaneous, and could be followed by a transamination catalyzed by Orf1 of the pmrF cluster (18), which is related to a large family of transaminases. The product of Orf1 would be the novel sugar nucleotide, UDP-L-4-aminoarabinose (designated L-AraN4 in Fig. 11).

The least obvious feature of the proposed pathway is the involvement of a bactoprenol-linked intermediate (Fig. 11), a possibility that is suggested by the sequence of the pmrF gene product (18). The latter shows significant similarity to dolichol-phosphomannose synthase of yeast (57), an enzyme that generates a key substrate required for protein glycosylation. The sequence similarity of pmrF to dolichol-phosphomannose syn-
an integral membrane protein. Orf1, 3, and 5 are encoded by genes that are clustered together with pmrF on the chromosomes of E. coli and S. typhimurium (18). Other open reading frames in this cluster (18) cannot yet be assigned a specific function. Presumably, many or all of these genes are activated by exposure of E. coli cells to \( \text{NH}_4\text{VO}_3 \), but they are expressed (under the control of the \( \text{phoP/Q} \) and \( \text{pmrA/B} \) systems) in the absence of \( \text{NH}_4\text{VO}_3 \) in Salmella. An intriguing observation is the fact (not shown) that Orf3 (18) contains what appears to be a second catalytic domain with similarity to formyl transferases. While the function of a formyl transferase in the above scheme is not immediately apparent, the possibility that a formyl moiety might be attached, perhaps transiently, to the amino group of \( \text{L-4-aminoarabinose} \) (designated \( \text{L-Ara}4\text{N} \)) deserves consideration.

The proposed involvement of the novel intermediate bactoprenol phosphoarabinose in lipid A modification raises the interesting possibility that \( \text{L-4-aminoarabinose} \) transfer to lipid A occurs on the periplasmic surface of the inner membrane (Fig. 11). Many enzymatic systems that utilize bactoprenol phosphate derivatives, such as some O-antigen polymerases and certain peptidoglycan glycosyltransferases, function in the periplasm (1, 58, 59). We have not yet identified a gene that might encode the putative bactoprenol phosphoarabinose flippase (Fig. 11), but we suggest that Orf5 of the pmrF cluster (18) might be the glycosyltransferase that attaches the \( \text{L-4-aminoarabinose} \) to lipid A (Fig. 11). This possibility is based on the observation that Orf5 displays distant sequence similarity to mannosyl transferases and that Orf5 is expressed (under the control of the \( \text{phoP/Q} \) and \( \text{pmrA/B} \) systems) in the absence of \( \text{NH}_4\text{VO}_3 \) in Salmella. An intriguing observation is the fact (not shown) that Orf3 (18) contains what appears to be a second catalytic domain with similarity to formyl transferases. While the function of a formyl transferase in the above scheme is not immediately apparent, the possibility that a formyl moiety might be attached, perhaps transiently, to the amino group of \( \text{L-4-aminoarabinose} \) (designated \( \text{L-Ara}4\text{N}4\text{N} \)) deserves consideration.

Acknowledgments—We thank Dr. George R. Dubay of the Duke University, Department of Chemistry, for the FAB mass spectrometry, and Drs. Garry D. Dotson, Anthony A. Ribeiro, and Amina S. Woods for assistance with NMR and MALDI/TOF experiments.

REFERENCES

1. Raetz, C. R. H. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., ed) Vol. 1, Second Ed., pp. 1035–1063, American Society for Microbiology, Washington, D.C.
2. Ritschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Leppnow, H., Ulmer, A. J., Zahringer, U., Seydel, U., Di Padova, P., Schreier, M., and Brade, H. (1994) \( \text{FASEB J.} \) 8, 217–225
3. Schnaitman, C. A., and Klena, J. D. (1993) \( \text{Microbiol. Rev.} \) 57, 655–682
4. Heinrichs, D. E., Yethon, J. A., and Whifield, C. (1998) Mol. Microbiol. 30, 221–232
5. Whitfield, C. (1995) Trends Microbiol. 3, 178–185
6. Morrison, D. C., and Ryan, J. L. (eds) (1992) Bacterial Endotoxic Lipopolysaccharides, Vol. I: Molecular Biochemistry and Cellular Biology, CRC Press, Boca Raton, FL
7. Nikaido, H. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., ed) Vol. 1, Second Ed., pp. 29–47, American Society for Microbiology, Washington, D.C.
8. Raetz, C. R. H. (1990) \( \text{Annu. Rev. Biochem.} \) 59, 129–170
9. Raetz, C. R. H. (1983) \( \text{J. Bacteriol.} \) 155, 5745–5753
10. Groisman, E. A., Kayser, J., and Soncini, F. C. (1997) \( \text{J. Bacteriol.} \) 179, 7040–7045
11. Cotter, R. J., Honovich, J., Qureshi, N., and Takayama, K. (1987) Biomed. Environ. Mass Spectrom. 14, 591–598
12. Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., and Raetz, C. R. H. (1998) \( \text{J. Biol. Chem.} \) 273, 12466–12475
13. Wyckoff, T. J. O., Raetz, C. R. H., and Jackman, J. E. (1998) \( \text{Trends Microbiol.} \) 6, 154–159
14. Babinski, K. J., and Raetz, C. R. H. (1998) \( \text{FASEB J.} \) 12, A1288
15. Karbians, D., Deprun, C., and Caroff, M. (1993) \( \text{J. Bacteriol.} \) 175, 2988–2993
16. Guo, L., Lim, K. B., Gun, S. J., Rainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) \( \text{Science} \) 276, 250–252
17. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gun, S. J., Hackett, M., and Miller, S. I. (1997) \( \text{Cell} \) 89, 189–198
18. Gun, S. J., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S. I. (1998) Mol. Microbiol. 27, 1171–1182
19. Groisman, E. A., Kayser, J., and Soncini, F. C. (1997) \( \text{J. Bacteriol.} \) 179, 7040–7045
20. Helder, M. H., Tung, J., Barrilay, L., and Khorana, H. G. (1979) \( \text{J. Biol. Chem.} \) 254, 5906–5917
21. Sidorczyk, Z., Zahringer, U., and Raets, T. E. (1983) \( \text{Eur. J. Biochem.} \) 129, 481–487
22. Meyers, E., Parker, W. L., Brown, W. E., Linnett, P., and Strominger, J. L. (1974) Annu. N. Y. Acad. Sci. 235, 493–501

---

\( ^7 \) K. A. White, Z. Zhou, and C. R. H. Raetz, unpublished data.
