JOURNAL OF BACTERIOLOGY, Apr. 2005, p. 2448–2457 Vol. 187, No. 7
0021-9193/05/$08.00+0 doi:10.1128/JB.187.7.2448–2457.2005


# Inhibition of *Salmonella enterica* Serovar Typhimurium Lipopolysaccharide Deacylation by Aminoarabinose Membrane Modification

Kiyoshi Kawasaki,[1,2] Robert K. Ernst,[3] and Samuel I. Miller[1,3,4]*

*Departments of Microbiology,[1] Medicine,[3] and Genome Sciences,[4] University of Washington, Seattle, Washington, and Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan[2]*

Received 27 September 2004/Accepted 21 December 2004

***Salmonella enterica*** **serovar Typhimurium remodels the lipid A component of lipopolysaccharide, a major component of the outer membrane, to survive within animals. The activation of the sensor kinase PhoQ in host environments increases the synthesis of enzymes that deacylate, palmitoylate, hydroxylate, and attach aminoarabinose to lipid A, also known as endotoxin. These modifications promote bacterial resistance to antimicrobial peptides and reduce the host recognition of lipid A by Toll-like receptor 4. The *Salmonella* lipid A 3-*O*-deacylase, PagL, is an outer membrane protein whose expression is regulated by PhoQ. In *S. enterica* serovar Typhimurium strains that had the ability to add aminoarabinose to lipid A, 3-*O*-deacylated lipid A species were not detected, despite the PhoQ induction of PagL protein expression. In contrast, strains defective for the aminoarabinose modification of lipid A demonstrated in vivo PagL activity, indicating that this membrane modification inhibited PagL's enzymatic activity. Since not all lipid A molecules are modified with aminoarabinose upon PhoQ activation, these results cannot be ascribed to the substrate specificity of PagL. PagL-dependent deacylation was detected in sonically disrupted membranes and membranes treated with the nonionic detergent *n*-octyl-β-D-glucopyranoside, suggesting that perturbation of the intact outer membrane releases PagL from posttranslational inhibition by aminoarabinose-containing membranes. Taken together, these results suggest that PagL enzymatic deacylation is posttranslationally inhibited by membrane environments, which either sequester PagL from its substrate or alter its conformation.**

Lipopolysaccharide (LPS) is the major constituent of the gram-negative bacterial outer membrane. LPS consists of a hydrophobic domain known as lipid A, which comprises the outer leaflet of the outer membrane, and a nonrepeating core oligosaccharide coupled to a distal polysaccharide (O antigen), which extends from the bacterial surface (reviewed in reference 33). Lipid A, also known as endotoxin, is the bioactive component of LPS and is involved in bacterial virulence through its recognition by the Toll-like receptor 4 complex (25, 31, 32, 35). The structure of lipid A is relatively conserved among different enteric bacteria. The lipid A domain of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium is a β-1′,6-linked disaccharide of glucosamine which is phosphorylated at the 1 and 4′ positions and acylated at the 2, 3, 2′, and 3′ positions with 3-hydroxymyristate (3-OH $C_{14:0}$). The hydroxy groups of the 3-OH $C_{14:0}$ chains attached at positions 2′ and 3′ are further acylated with laurate ($C_{12:0}$) and myristate ($C_{14:0}$), respectively (reviewed in reference 33) (Fig. 1).

In response to environmental conditions, including host microenvironments, *S. enterica* serovar Typhimurium covalently modifies its lipid A by palmitoylation, deacylation, the formation of a 2-hydroxymyristate group (hydroxylation), and the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (reviewed in references 5 and 33) (Fig. 1). Similar modifications also occur in other gram-negative bacteria, including *E. coli*, *Yersinia enterocolitica*, and *Pseudomonas aeruginosa* (6, 15, 43). Modified lipid A promotes bacterial survival by increasing the resistance to antimicrobial peptides and by altering the host recognition of LPS (5). Genes that promote these modifications are essential for virulence in a variety of pathogens. Lipid A modifications require the activation of the two-component regulatory system PhoP-PhoQ (14), which is essential for *Salmonella* virulence. PhoQ is a sensor histidine kinase that responds to environmental conditions, including those within mammalian tissues and macrophage phagosomes and those that destabilize the bacterial membrane, such as magnesium-limited growth medium and exposure to antimicrobial peptides (1, 9, 37). In response to specific environmental signals, PhoQ phosphorylates PhoP, leading to the activation or repression of >40 different genes (2, 10, 19, 26, 27). These include the activation of *pagP*, which encodes a lipid A palmitoyltransferase (3, 15), and *pagL*, which encodes a lipid A 3-*O*-deacylase (39). The L-Ara4N attachment to lipid A requires the activation of a second two-component regulatory system, PmrA-PmrB, which regulates the expression of the *pmrF* operon (also known as the *pbgP*/*E* operon) and *pmrE* (also known as *ugd*). These genes are required for L-Ara4N synthesis and its transfer to lipid A (11, 12, 40). PmrA-PmrB-regulated lipid A modifications are required for the maximal virulence of *S. enterica* serovar Typhimurium infection for BALB/c mice by the oral route (12). The activation of PhoP-PhoQ leads to the transcriptional activation of *pmrAB*, result-

* Corresponding author. Mailing address: Departments of Medicine, Microbiology, and Genome Sciences, University of Washington, Health Sciences Building, K140, Box 357710, 1959 Pacific St. N.E., Seattle, WA 98195. Phone: (206) 616-5107. Fax: (206) 616-5109. E-mail: millersi@u.washington.edu.

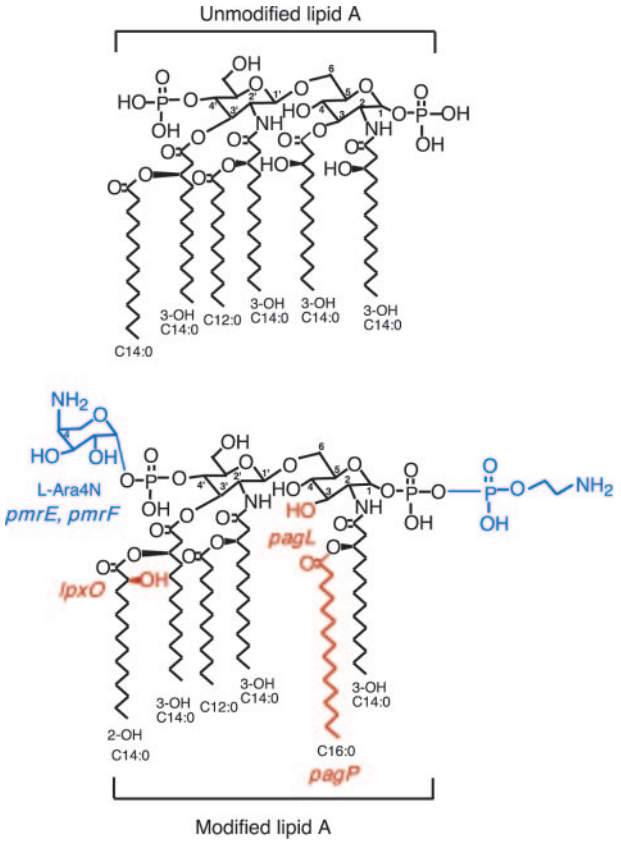


FIG. 1. PhoP-PhoQ- and PmrA-PmrB-regulated lipid A modifications in *S. enterica* serovar Typhimurium. The phosphate residues and acyl chains of lipid A in *S. enterica* serovar Typhimurium can be derivatized in a PhoP-PhoQ- and PmrA-PmrB-regulated manner (reviewed in reference 5). Phosphate residues can be attached with L-Ara4N and/or phosphoethanolamine groups (shown in blue), both of which are under the control of PmrA-PmrB (11, 44). Minor species were present in which the locations of the L-Ara4N and phosphoethanolamine groups were reversed or in which both phosphates were modified with the same substituent (44). *pmrE* (also known as *ugd*) is predicted to encode a UDP-glucose dehydrogenase. The *pmrF* locus is an operon (*pmrHFIJKLM*) that carries seven open reading frames which are predicted to encode other enzymes involved in L-Ara4N synthesis and L-Ara4N transfer to lipid A (12, 40). Both the *pmrE* and *pmrF* loci are necessary for the PmrA-PmrB-regulated L-Ara4N attachment to lipid A (11). The addition of the palmitate chain is catalyzed by PagP (3, 15), the formation of the 2-hydroxymyristate group requires LpxO (8), and deacylation at the 3 position of lipid A is catalyzed by PagL (39) (shown in red). The *pagL* and *pagP* genes and lipid A hydroxylation are regulated by PhoP-PhoQ (2, 8). PhoP-PhoQ also activates PmrA-PmrB; therefore, the L-Ara4N and phosphoethanolamine modifications occur under PhoP-PhoQ-activating conditions (14, 43).

ing in the activation of the PmrA-PmrB regulon (13). This activation is mediated by *pmrD* (20). Therefore, conditions that activate PhoP-PhoQ promote lipid A modifications, including those regulated by PmrA-PmrB (Fig. 1).

Previous results demonstrated that 3-*O*-deacylated lipid A species have a reduced ability to induce cellular signaling through Toll-like receptor 4 (18). Although *pagL* expression is induced by the activation of PhoP-PhoQ (2, 39), significant lipid A deacylation was not observed under standard PhoP-PhoQ-activating growth conditions (39). These observations suggested that an unknown factor(s) prevents lipid A deacylation by PagL. Here we report that 3-*O*-deacylation could be detected in strains harboring a mutation in *pmrA*, *pmrE*, or *pmrF*, indicating that an L-Ara4N modification inhibits lipid A deacylation by PagL. These findings suggest that the outer membrane structure and/or charge, dependent on L-Ara4N modification, may regulate the activity of PagL.

## MATERIALS AND METHODS

**Materials** All chemicals were reagent grade or better. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.) and Invitrogen (Carlsbad, Calif.). Oligonucleotides were prepared commercially by Invitrogen. [$^{32}$P]orthophosphate was purchased from Perkin-Elmer (Boston, Mass.). Instant methanolic HCl kits were obtained from Alltech (Deerfield, Ill.). Silica gel 60 high-performance thin-layer chromatography (HPTLC) plates were purchased from EM Science (Gibbstown, N.J.). Triton X-100 was purchased from Wako Pure Chemical Industries (Osaka, Japan), and *n*-octyl-β-D-glucopyranoside was purchased from Sigma-Aldrich Japan (Tokyo, Japan).

**Bacterial strains and growth conditions.** The bacterial strains and plasmids used for this study are listed in Table 1. *S. enterica* serovar Typhimurium strain CS019 (26), a derivative of 14028s (American Type Culture Collection, Manassas, Va.), was used as the wild-type strain in this study.

Bacteria were grown at 37°C with aeration in N minimal medium [5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM $K_2SO_4$, 1 mM $KH_2PO_4$, 0.28% glycerol (vol/vol), 0.1% Casamino Acids (wt/vol), 2 mg of thiamine/liter, and 0.1 M Tris-HCl (pH 7.4)] (7, 28). The growth medium was supplemented with 10 mM or 10 μM $MgCl_2$. The growth medium containing 10 μM $MgCl_2$ was used as $Mg^{2+}$-limited growth medium. Ampicillin (10 μg/ml) was used for the cultivation of strains transformed with plasmids. Bacterial colonies were picked and grown overnight in growth medium containing 10 mM or 10 μM $MgCl_2$. The overnight cultures were diluted 1:100 in the growth medium containing 10 mM $MgCl_2$ or 1:10 in the growth medium containing 10 μM $MgCl_2$ and then grown for 24 h. After cultivation, stationary-phase cells were collected and used for further analysis.

**Bacterial genetic and molecular biology techniques.** Phage P22-mediated transductions were performed as described previously (4). Plasmid DNAs were introduced into bacterial strains by electroporation with *E. coli* Pulser (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. Recombinant DNA techniques were performed according to standard protocols (36).

The *pmrE* coding region was amplified from pJG02 (11) by a PCR with *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, Calif.). The primers used for PCR were KK4 (5′-GGTCTCGAGAGCTGGAGACAGTGTAGCCA-3′) and KK5 (5′-CTTGAATTCTTTCTGCAAAAATGTTTAAGCCCGG-3′). The amplified DNA fragment was cloned into EcoRI and XhoI sites of the low-copy-number vector pWKS30 (41), and the resulting plasmid construct was named pWKS30-*pmrE*. For construction of a plasmid that expressed a recombinant PagL protein bearing a six-His epitope at the C terminus, the *pagL* gene was amplified by PCR from pWLP21 (39). The primers used for PCR were WLP22-EcoR1 (39) and pagL-Cterm-His6 (5′-CGCGGATCCTCAGTGGTGGTGGTGGTGGTGGAA ATTATAACTAATTGA-3′). The amplified DNA fragment was cloned into EcoRI and BamHI sites of pWKS30, and the resulting construct was named pWKS30-*pagL*-His6. The inserts of the plasmid constructs were verified by sequencing.

**Lipid A preparation.** For mass spectrometry, lipid A was purified as described previously (42). In brief, cells collected from a 25-ml culture were resuspended in 500 μl of Tri-reagent (Molecular Research Center Inc., Cincinnati, Ohio). After incubation for 30 min at room temperature, 100 μl of chloroform was added. After 15 min, the mixture was centrifuged and the aqueous phase was recovered. LPS was extracted three times by the addition of 500 μl of water to the organic phase, and the aqueous phase containing LPS was lyophilized. Five hundred microliters of 10 mM sodium acetate buffer (pH 4.5) containing 1% sodium dodecyl sulfate (SDS) was added to the lyophilized LPS, and the LPS was hydrolyzed to remove sugar chains from lipid A by incubation at 100°C for 1 h (34), followed by lyophilization. The lyophilized lipid A was washed one time with 0.02 N HCl in 95% ethanol, followed by three washes with 95% ethanol. The washed lipid A was lyophilized and used for mass spectrometry analysis. For thin-layer chromatography analysis, preparations of lipid A were performed as described previously (43).

**Mass spectrometry.** Samples were dissolved in 20 mg of 5-chloro-2-mercaptobenzothiazole matrices/ml in chloroform-methanol (1:1 [vol/vol]). The mix-

TABLE 1. *Salmonella* strains and plasmids used for this study

| Strain or plasmid | Description | Reference |
|---|---|---|
| *S. enterica* serovar Typhimurium strains | | |
| CS019 | ATCC 14028s *phoN2 zxx*::*6251*Tn*10d*-Cam (wild type) | 26 |
| CS283 | CS019 *pagL1*::Tn*phoA* (previously named CS993) | 2 |
| JSG421 | ATCC 14028s *pmrA*::Tn*10d* | 13 |
| KCS039 | CS019 *pmrA*::Tn*10d* | This work |
| KCS040 | CS019 *pmrA*::Tn*10d pagL1*::Tn*phoA* | This work |
| CS401 | CS019 Strep$^r$ | Miller laboratory |
| CS586 | CS019 Strep$^r$ Δ*pagL* | 39 |
| KCS042 | CS019 *pmrA*::Tn*10d* Strep$^r$ | This work |
| KCS043 | CS019 *pmrA*::Tn*10d* Strep$^r$ Δ*pagL* | This work |
| CS287 | CS019 *pagP1*::Tn*phoA* (previously named CS1247) | 2 |
| JSG486 | ATCC 14028s *pmrA505 zjd*::Tn*10d*-Cam *pmrE1*::Tn*10d* | 11 |
| KCS041 | ATCC 14028s *pmrE1*::Tn*10d* | This work |
| KCS044 | CS019 *pmrE1*::Tn*10d* | This work |
| KCS045 | CS019 *pmrE1*::Tn*10d pagL1*::Tn*phoA* | This work |
| JSG485 | ATCC 14028s *pmrA505 zjd*::Tn*10d*-Cam *pmrF1*::Tn*10d* | 11 |
| KCS048 | ATCC 14028s *pmrF1*::Tn*10d* | This work |
| KCS049 | CS019 *pmrF1*::Tn*10d* | This work |
| KCS050 | CS019 *pmrF1*::Tn*10d pagL1*::Tn*phoA* | This work |
| Plasmids | | |
| pWKS30 | Low-copy-number cloning vector | 41 |
| pWKS30-*pmrE* | pWKS30 containing *pmrE*, including the coding region and flanking 236-bp upstream and 56-bp downstream regions | This work |
| pWKS30-*pagL*-His6 | pWKS30 containing *pagL*, including the coding region for PagL bearing six His residues at the C terminus and the 79-bp upstream region | This work |

tures were allowed to dry at room temperature on the sample plate prior to mass spectrometry analysis. Spectra were obtained in the negative reflection mode by use of a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) Bruker BiflexIII mass spectrometer (Bruker Daltonics, Inc., Billerica, Mass.) or a Voyager-DE STR mass spectrometer (Applied Biosystems Japan, Tokyo, Japan). Each spectrum was an average of 200 shots.

**Thin- layer chromatography.** HPTLC plates were developed in chloroform–pyridine–88% formic acid–methanol–water (60:35:10:5:2 [vol/vol]). $^{32}$P-labeled spots were visualized with a STORM840 phosphorimager and ImageQuant software (Molecular Dynamics). For the identification of lipid A spots, unlabeled lipid A was developed on HPTLC plates as described above. Spots of unlabeled lipid A species were visualized by spraying water on HPTLC plates. The visualized spots were extracted from silica as described previously (43) and used for identification of the species by mass spectrometry.

**Analysis of LPS fatty acids.** LPS isolation and an analysis of its fatty acids were performed as described previously (38), with slight modifications. In brief, cells grown in 25 ml of the growth medium containing 10 μM $MgCl_2$ were collected and lyophilized. For LPS purification, the lyophilized cells were extracted three times with 90% phenol at 70°C, and the cooled aqueous layers were recovered. After extraction with diethyl ether, the aqueous phase was lyophilized. LPS fatty acids were derivatized to fatty acid methyl esters by methanolysis in 2 M methanolic HCl at 90°C for 18 h, with the addition of pentadecanoic acid as an internal standard. After the addition of an equal volume of a saturated NaCl solution, the methyl esters were extracted into hexane and analyzed by capillary gas chromatography. The amounts of $C_{12:0}$, $C_{14:0}$, 2-hydroxymyristate (2-OH $C_{14:0}$), 3-OH $C_{14:0}$, and $C_{16:0}$ were determined.

**Alkaline phosphatase assay.** Measurements of alkaline phosphatase activities and calculations of their units were performed as described previously (2).

**Membrane preparation.** All steps were performed at 4°C or on ice. Cells collected from a 500-ml culture were suspended in 1 ml of 50 mM HEPES, pH 7.5, and then sonically disrupted four times for 15 s each time at 1-min intervals at setting 3 of a Virsonic 450 sonicator (VirTis, Garginer, N.Y.). The crude lysate was cleared by centrifugation at 7,000 × *g* for 15 min. Membranes were precipitated by centrifugation at 149,000 × *g* for 60 min and were resuspended in 50 mM HEPES, pH 7.5, at a protein concentration of ~10 mg/ml. Protein concentrations were determined by use of the bicinchoninic acid protein assay reagent (Pierce, Rockford, Ill.), with bovine serum albumin as a standard.

**SDS-polyacrylamide gel electrophoresis and Western blotting.** Proteins were fractionated by SDS–12.5% polyacrylamide gel electrophoresis under reducing conditions (21). Molecular mass standards were purchased from Invitrogen. Proteins separated in the gel were stained with Coomassie blue. For Western blot analysis, proteins separated in the gel were electroblotted onto a polyvinylidene difluoride membrane in 25 mM Tris–192 mM glycine–0.02% SDS–20% methanol at 22 V/cm for 60 min. The blot was incubated with anti-tetra-His antibodies (QIAGEN, Valencia, Calif.) and subsequently incubated with anti-mouse immunoglobulin G linked to horseradish peroxidase. Cross-reactive proteins were detected with ECL Plus Western blotting detection reagents (Amersham Biosciences, Piscataway, N.J.).

**Sonic disruption of *Salmonella*.** Cell disruption was performed on ice by use of a Virsonic 450 sonicator (VirTis) on setting 2 or a W-225R ultrasonic disruptor (Heat Systems Ultrasonics) on setting 3. Bacteria were sonicated three times for 10 s each at 1-min intervals.

**Detergent treatment of *Salmonella*.** Cells were cultivated in growth medium containing 10 μM $MgCl_2$. Cells collected from a 25-ml culture were suspended in 200 μl of 50 mM HEPES (pH 7.5) containing 250 mM NaCl and 1% (wt/vol) *n*-octyl-β-D-glucopyranoside or 1% (vol/vol) Triton X-100. Additional sonication of Triton X-100-treated cells was performed with a W-225R ultrasonic disruptor. The cells were incubated at 37°C for 1 h, and their lipid A species were analyzed by use of a Voyager-DE STR mass spectrometer.

**EDTA treatment of *Salmonella*.** Strains CS401 and CS586 were cultivated in growth medium containing 10 μM $MgCl_2$. Cells collected from 25-ml cultures were suspended in 200 μl of 120 mM Tris-HCl (pH 8.0) containing 5 or 10 mM EDTA. The cells were incubated at 37°C for 1 h, and their total lipid A species were analyzed by use of a Voyager-DE STR mass spectrometer.

## RESULTS

**Deacylation of lipid A is not detectable in *S. enterica* serovar Typhimurium cultivated in magnesium-limited medium.** The PhoP-PhoQ-activated gene *pagL* encodes a lipid A deacylase that removes a 3-OH $C_{14:0}$ moiety from position 3 of lipid A (Fig. 1). Membranes harvested from *S. enterica* serovar Typhimurium strains in which PhoP is constitutively active have robust PagL activity (39). However, PagL-dependent in vivo deacylation of lipid A was not observed (39). Since $Mg^{2+}$-limited growth medium activates transcription of the PhoP-PhoQ regulon (7), wild-type salmonellae were grown in $Mg^{2+}$-

limited medium, and lipid A was assayed for PagL activity by MALDI-TOF mass spectrometry. Although other modified lipid A species, including palmitoylated (*m/z* 2036, 2052, and 2184), hydroxylated (*m/z* 1814, 1945, 2052, and 2184), and L-Ara4N-attached (*m/z* 1945 and 2184) species, were detected in the lipid A preparation by MALDI-TOF mass spectrometry (Fig. 2B and Table 2), deacylated species were not detected. These results were confirmed by an analysis of $^{32}$P-labeled lipid A species by thin-layer chromatography (see below and Fig. 3). Therefore, similar to the results observed with PhoP constitutive mutants, no PagL activity was observed when bacteria were grown under growth conditions that activate PhoP-PhoQ.

**3-*O*-Deacylation of lipid A is promoted in strains harboring a mutation in *pmrA*, a transcriptional regulator that promotes the addition of L-Ara4N and phosphoethanolamine.** The above results suggested that the posttranscriptional regulation of PagL was responsible for the lack of lipid A deacylation. We hypothesized that structural changes in the outer membrane were responsible for this effect. A two-component regulatory system encoded by *pmrAB* promotes the modification of lipid A and core oligosaccharide phosphate residues with L-Ara4N and phosphoethanolamine (5, 11, 13, 16). The PmrA-PmrB regulon was a plausible candidate for inhibition of lipid A deacylation, since it is also activated by PhoP-PhoQ and its expression alters the bacterial cell surface charge (reviewed in reference 5). To test this possibility, we analyzed a strain with a *pmrA*-null mutation for the ability to deacylate lipid A during cultivation in $Mg^{2+}$-limited medium. Deacylated lipid A species (*m/z* 1571, 1587, and 1826) were detected from a *pmrA*-null mutant strain harboring the *pmrA*::Tn*10d* transposon insertion (PmrA$^-$) (Fig. 2C and Table 2). As expected, deacylated lipid A species were not observed in lipid A isolated from a mutant strain harboring both the *pmrA*::Tn*10d* and *pagL*::Tn*phoA* alleles (PmrA$^-$ PagL$^-$), indicating that the lipid A deacylation was PagL dependent (Fig. 2D). To confirm that lipid A deacylation was PagL dependent, we analyzed a strain that harbors both *pmrA*-null and nonpolar *pagL* deletion alleles (PmrA$^-$ ΔPagL). Deacylated lipid A species were not observed in lipid A from the *pmrA* Δ*pagL* mutant (Fig. 2E and F), indicating that the lipid A deacylation observed in the *pmrA*-null mutant was PagL dependent. $^{32}$P-labeled lipid A was prepared from a *pmrA* mutant cultivated in $Mg^{2+}$-limited medium and then was analyzed by thin-layer chromatography. Consistent with the results of MALDI-TOF mass spectrometry, lipid A prepared from the *pmrA* mutant showed specific radiolabeled spots corresponding to deacylated lipid A species (*m/z* 1571 and 1587) (Fig. 3 and Table 2). In contrast, lipid A prepared from wild-type or *pmrA pagL* mutant salmonellae did not show the deacylated lipid A species (Fig. 3), indicating that wild-type *pmrA* interferes with PagL-dependent deacylation.

To further characterize the effect of a *pmrA*-null mutation, we analyzed the fatty acid compositions of LPS prepared from wild-type, *pmrA* mutant, and *pmrA pagL* mutant *Salmonella* strains cultivated in $Mg^{2+}$-limited medium. The relative contents of 3-OH $C_{14:0}$ in LPS prepared from wild-type, *pmrA* mutant, and *pmrA pagL* mutant salmonellae were 3.59, 3.05, and 3.67 (molar ratios), respectively (Table 3). A decrease in the 3-OH $C_{14:0}$ content was observed for the *pmrA* mutant and was PagL dependent. Taken together, these results indicate that functions activated by PmrA-PmrB inhibit the deacylation of lipid A.

In addition, we analyzed the *pagP*-null mutant for the ability to deacylate lipid A during cultivation in $Mg^{2+}$-limited medium. Deacylated lipid A species were not detected from a *pagP*-null mutant strain harboring the *pagP*::Tn*phoA* allele by MALDI-TOF mass spectrometry (data not shown), suggesting that lipid A palmitoylation was not involved in the inhibition of lipid A deacylation.

**PmrA-PmrB-regulated genes necessary for L-Ara4N attachment to lipid A also interfere with lipid A 3-*O*-deacylation by PagL.** PmrA-PmrB regulates outer membrane modifications, including the addition of L-Ara4N and phosphoethanolamine to lipid A and core oligosaccharide phosphate residues (Fig. 1). However, PmrA-PmrB may regulate additional proteins and cellular functions. Therefore, mutations in PmrA-PmrB-regulated genes were analyzed for an effect on PagL-dependent deacylation. *pmrE* and *pmrF* are required for L-Ara4N attachment to lipid A (11). Lipid A was prepared from strains that harbored either a *pmrE*::Tn*10d* (PmrE$^-$) or *pmrF*::Tn*10d* (PmrF$^-$) allele and then was analyzed by MALDI-TOF mass spectrometry. In both lipid A preparations, deacylated lipid A species (*m/z* 1587/1588 and 1826) were detected (Fig. 2G and I). In contrast, the deacylated species were not detected in lipid A prepared from the *pmrE pagL* and *pmrF pagL* mutants (Fig. 2H and J), indicating that the deacylation observed was PagL dependent. The deacylated lipid A species were not detected in lipid A from the *pmrE* mutant transformed with pWKS30-*pmrE*, a low-copy-number vector containing the wild-type *pmrE* gene (Fig. 2K and L), indicating that the inhibition of deacylation in a *pmrE* mutant could be complemented by a wild-type copy of the gene. Consistent with these results, deacylated lipid A species were detected by thin-layer chromatography of $^{32}$P-labeled lipid A prepared from *pmrE*-null and *pmrF*-null mutants, and they were not detected in lipid A prepared from *pmrE pagL*-null and *pmrF pagL*-null mutants (Fig. 3). The relative contents of 3-OH $C_{14:0}$ in LPS prepared from *pmrE*, *pmrE pagL*, *pmrF*, and *pmrF pagL* mutant salmonellae were 3.23, 3.74, 3.12, and 3.62 (molar ratios), respectively (Table 3). A PagL-dependent decrease in 3-OH $C_{14:0}$ was observed for both the *pmrE* and *pmrF* mutants. Taken together, these results indicate that the expression of *pmrE* and *pmrF*, both of which are necessary for L-Ara4N attachment to lipid A, interferes with PagL-dependent lipid A 3-*O*-deacylation. It is noteworthy that many lipid A species (*m/z* 1798, 1814, 2036, and 2052 in Fig. 2B) that were not modified with L-Ara4N were present in lipid A prepared from the wild-type strain, suggesting that the lack of lipid A deacylation could not be simply ascribed to the substrate specificity of PagL. In addition, our results do not exclude the possibility that other PmrA-PmrB-regulated outer membrane modifications, in addition to L-Ara4N modification, also contribute to the inhibition of PagL-dependent deacylation.

**Lipid A deacylation by PagL is posttranslationally regulated and is not related to the abundance or stability of the enzyme.** It was possible that an alteration of the outer membrane promoted the stability or abundance of PagL within the membrane. The expression of PagL was examined by measuring the alkaline phosphatase activity of a *pagL*::*phoA* fusion in strains that harbored a wild-type or null *pmrA* gene. PagL expression

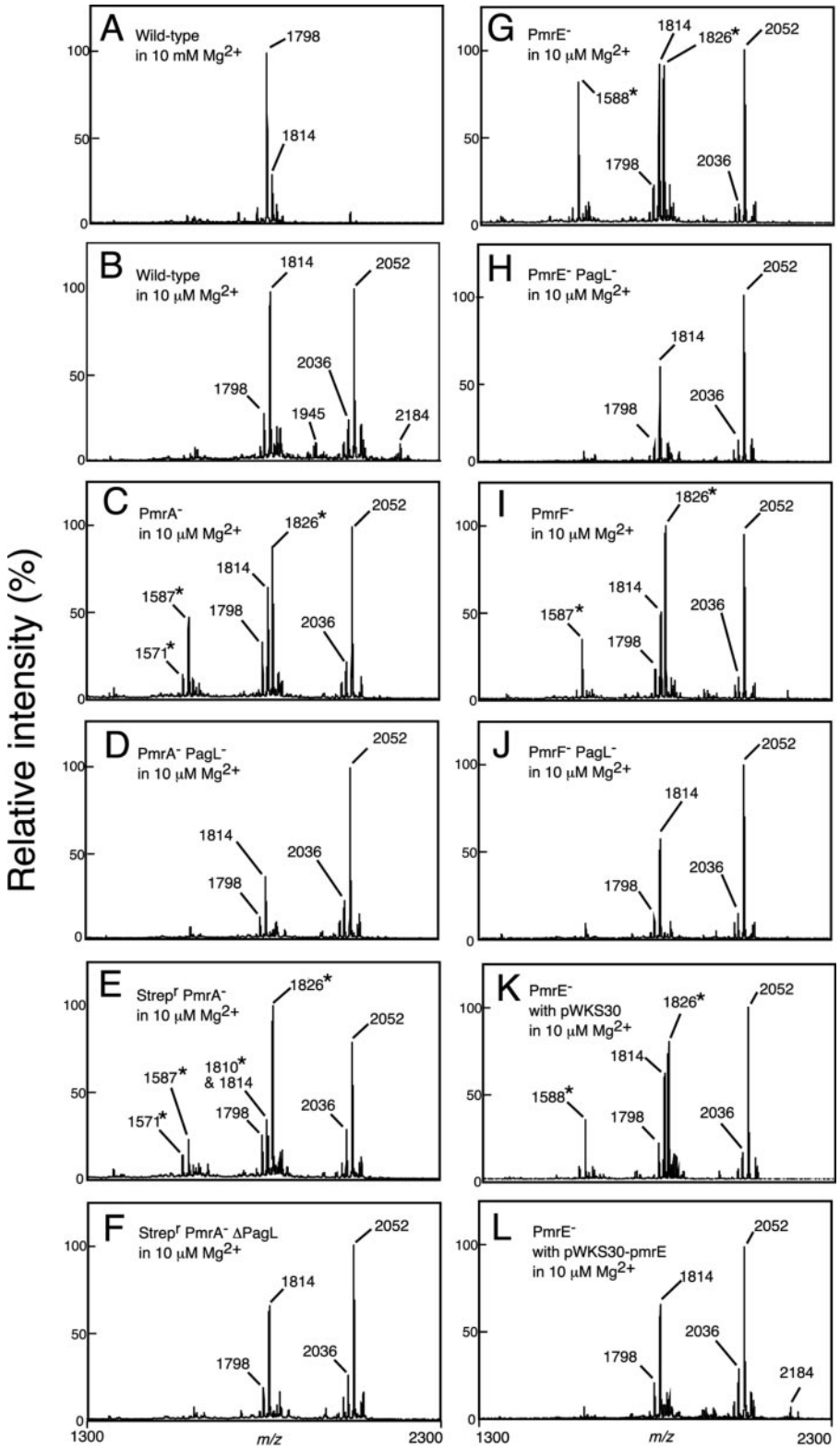


FIG. 2. MALDI-TOF mass spectrometry of lipid A purified from mutant *S. enterica* serovar Typhimurium strains. Strains were cultivated in growth medium containing 10 mM $MgCl_2$ (A) or 10 μM $MgCl_2$ (B to L). Lipid A species prepared from CS019 (A and B), KCS039 (C), KCS040 (D), KCS042 (E), KCS043 (F), KCS044 (G), KCS045 (H), KCS049 (I), KCS050 (J), KCS044 transformed with pWKS30 (K), and KCS044 transformed with pWKS30-*pmrE* (L) were analyzed by use of a Bruker BiflexIII mass spectrometer. For the cultivation of transformants, 10 μg of ampicillin/ml was used (K and L). *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are marked with asterisks. Structural interpretations of lipid A species are summarized in Table 2.

TABLE 2. Structural interpretations of lipid A species detected by mass spectrometry

| *m/z* | Lipid A modification(s)[a] |
|---|---|
| 1571 | Deacylation |
| 1587, 1588 | Deacylation and hydroxylation |
| 1798 | Unmodified |
| 1810 | Deacylation and palmitoylation |
| 1814 | Hydroxylation |
| 1826 | Deacylation, palmitoylation, and hydroxylation |
| 1945 | L-Ara4N attachment and hydroxylation |
| 2036 | Palmitoylation |
| 2052 | Palmitoylation and hydroxylation |
| 2184 | Palmitoylation, L-Ara4N attachment, and hydroxylation |

[a] Interpretations were based on previous work (11, 14, 39, 43).

levels were similar when both strains were cultivated in $Mg^{2+}$-limited medium (Table 4). These results suggested that PagL expression levels were not responsible for the difference in lipid A deacylation status between the strains harboring *pmrA*-null and wild-type alleles. To further address the relationship between PagL expression levels and lipid A deacylation, we introduced pWKS30-*pagL*-His6, a low-copy-number vector encoding a recombinant PagL protein that bears a six-His epitope

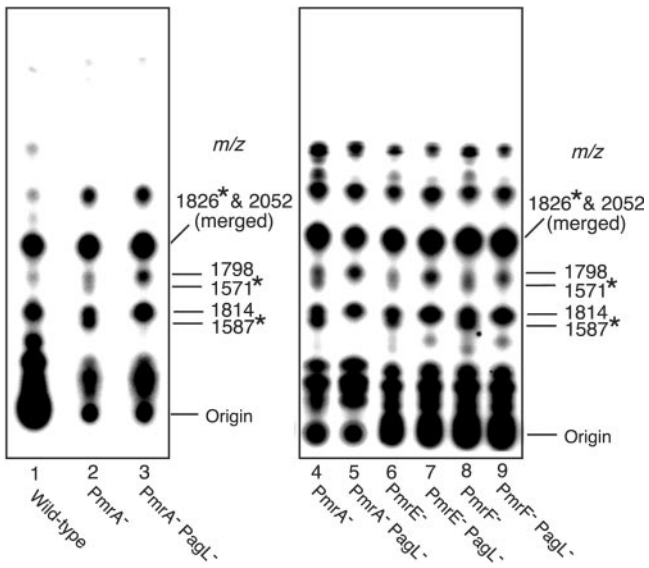


FIG. 3. Thin-layer chromatography analysis of $^{32}$P-labeled lipid A species purified from mutant *S. enterica* serovar Typhimurium strains. Strains were cultivated in growth medium containing 10 μM $MgCl_2$. [$^{32}$P]orthophosphate (10 μCi/ml) was added to the growth medium for the labeling of lipid A species. Lipid A species purified from CS019 (lane 1), KCS039 (lanes 2 and 4), KCS040 (lanes 3 and 5), KCS044 (lane 6), KCS045 (lane 7), KCS049 (lane 8), and KCS050 (lane 9) were spotted onto HPTLC plates and developed. To identify lipid A species, we prepared unlabeled lipid A species from KCS039 and KCS040 and cultivated them as described above, without [$^{32}$P]orthophosphate. Unlabeled lipid A species were developed by HPTLC, and lipid A species were visualized by spraying water on the plate. Lipid A species were extracted from the plate and analyzed by use of a Bruker BiflexIII mass spectrometer (data not shown). Major *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are marked with asterisks. Structural interpretations of lipid A species are summarized in Table 2.

at its C terminus, into *pagL* and *pagL pmrA* mutants. Lipid A was isolated from transformants cultivated in $Mg^{2+}$-limited medium and analyzed by MALDI-TOF mass spectrometry. Deacylated lipid A species (*m/z* 1587 and 1826) were produced by the *pagL pmrA* mutant transformed with pWKS30-*pagL*-His6, but not by the *pagL* mutant transformed with pWKS30-*pagL*-His6 (Fig. 4A), indicating that the histidine-tagged enzyme behaves similarly to the wild-type enzyme in vivo. The recombinant PagL-His protein was expressed at similar levels in both strains (Fig. 4B, lanes 4 and 5). Taken together, these results show that the PmrA-PmrB status did not grossly alter PagL expression levels and/or stability.

**PagL-dependent lipid A deacylation occurs in disrupted *Salmonella* membranes.** The above results indicated that PmrA-PmrB-induced outer membrane modifications, including the L-Ara4N modification of lipid A, inhibit lipid A deacylation by PagL. To address the requirement of an intact cell membrane for the observed inhibition, we examined whether deacylation could occur in disrupted membranes. A Δ*pagL* strain and its parental wild-type strains were cultivated in $Mg^{2+}$-limited medium. The incubation of sonically disrupted wild-type cells resulted in the production of deacylated lipid A species (*m/z* 1587 and 1826), and the deacylation was not detected in the *pagL*-null strain (Fig. 5). Furthermore, deacylated lipid A species (*m/z* 1571, 1588, and 1826) were detected in wild-type cells treated with 1% *n*-octyl-β-D-glucopyranoside, and the deacylation was PagL dependent (Fig. 6A, B, and C). In contrast, PagL-dependent lipid A deacylation was not observed in cells treated with 1% Triton X-100 (Fig. 6D). Sonication in the presence of 1% Triton X-100 resulted in the production of PagL-dependent deacylated lipid A species (*m/z* 1588 and 1826), indicating that Triton X-100 did not interfere with PagL activity (Fig. 6E and F). This result is consistent with the previous findings that lipid A 3-*O*-deacylation could be observed in PhoP-constitutive outer membranes under conditions that included 0.25% Triton X-100 and 10 mM EDTA (3) or 0.1% Triton X-100 (40). These results indicate that the enzyme is functional in vitro under these conditions and is localized to the outer membrane despite its lack of in vivo activity. In addition, cells were treated with Tris-HCl buffer containing 5 or 10 mM EDTA, a condition that destabilizes the outer membrane (23, 24, 30). Lipid A prepared from EDTA-treated cells was analyzed by MALDI-TOF mass spectrometry, and deacylated lipid A species were not observed (data not shown). Taken together, these results indicate that a complete disruption of the membrane or the specific effect of *n*-octyl-β-D-glucopyranoside can release PagL from posttranslational inhibition, while Triton X-100 and EDTA treatments do not repress the inhibition of PagL by L-Ara4N modification.

## DISCUSSION

In this work, we demonstrated that *S. enterica* serovar Typhimurium PagL-dependent lipid A 3-*O*-deacylation is inhibited by the L-Ara4N modification of lipid A. These findings cannot be simply ascribed to the substrate specificity of PagL, since many lipid A species that are not modified with L-Ara4N exist in the membrane of a wild-type strain even when grown under conditions that promote lipid A modifications. Our results also indicated that a disruption of the intact bacterial

TABLE 3. Fatty acid composition of *S. enterica* serovar Typhimurium LPS[a]

| Strain | Phenotype | Relative fatty acid content[b] (molar ratio of indicated species to $C_{12:0}$) | | | | |
|---|---|---|---|---|---|---|
| | | $C_{12:0}$ | $C_{14:0}$ | 2-OH $C_{14:0}$ | 3-OH $C_{14:0}$ | $C_{16:0}$ |
| CS019 | Wild type | 1.00 | 0.349 ± 0.024 | 0.745 ± 0.045 | 3.590 ± 0.180 | 0.612 ± 0.026 |
| KCS039 | PmrA$^-$ | 1.00 | 0.357 ± 0.024 | 0.711 ± 0.052 | 3.046 ± 0.276 | 0.741 ± 0.013 |
| KCS040 | PmrA$^-$ PagL$^-$ | 1.00 | 0.364 ± 0.016 | 0.743 ± 0.025 | 3.667 ± 0.077 | 0.730 ± 0.056 |
| KCS044 | PmrE$^-$ | 1.00 | 0.337 ± 0.007 | 0.735 ± 0.027 | 3.229 ± 0.126 | 0.700 ± 0.031 |
| KCS045 | PmrE$^-$ PagL$^-$ | 1.00 | 0.365 ± 0.011 | 0.737 ± 0.029 | 3.736 ± 0.074 | 0.73 ± 0.040 |
| KCS049 | PmrF$^-$ | 1.00 | 0.341 ± 0.019 | 0.736 ± 0.033 | 3.116 ± 0.048 | 0.750 ± 0.021 |
| KCS050 | PmrF$^-$ PagL$^-$ | 1.00 | 0.344 ± 0.014 | 0.734 ± 0.007 | 3.615 ± 0.063 | 0.686 ± 0.031 |

[a] LPS fatty acid preparations from strains cultivated in growth medium containing 10 μM $MgCl_2$ were sequentially analyzed by capillary gas chromatography.
[b] The results shown are averages ± standard deviations of three (CS019, KCS039, KCS040, KSC045, KSC049, and KSC050) or two (KCS044) independent cultures.

membrane by sonication or *n*-octyl-β-D-glucopyranoside treatment releases PagL from this inhibition. Therefore, L-Ara4N modification of the membrane likely alters the membrane localization or conformation of PagL to inhibit its activity.

The gram-negative bacterial outer membrane is unique. The inner leaflet is composed of phospholipids, while the outer leaflet, excluding the area occupied by outer membrane proteins, is completely composed of LPS (reviewed in reference 29). Lipid A is synthesized at the inner surface of the inner membrane and then transported to the outer leaflet of the outer membrane (reviewed in reference 33). L-Ara4N incorporation into lipid A takes place at the periplasmic side of the inner membrane (40), and lipid A deacylation by PagL occurs in the outer membrane of *S. enterica* serovar Typhimurium (39). L-Ara4N lipid A modifications reduce the negative charge of the bacterial membrane, resulting in increased bacterial resistance to cationic antimicrobial peptides, including polymyxin, by altering the binding of these peptides to the membrane (11). However, the specific contents of L-Ara4N-modified lipid A species, which can change the charge states of outer membrane phosphates, remain to be studied. The charge status of the outer membrane as well as a structural effect of the L-Ara4N modification on the outer membrane may affect the PagL conformation or binding to lipid A. Since all lipid A molecules are not modified, one must hypothesize that PagL is in an inactive conformation or that it is specifically sequestered into domains upon insertion into the bacterial outer membrane. An effect of the modification and diversity of lipid A may be an organization of the protein components of the membrane into domains analogous to eukaryotic lipid rafts. This hypothesis is consistent with the fact that chemical and structural features of lipid A are strikingly similar to those of glycosphingolipids, a major component of eukaryotic lipid rafts (reviewed in reference 29).

A precedent for the regulation of lipid A modification enzymes by the lipid A structure has been shown previously.

TABLE 4. Measurement of *pagL*::*phoA* fusion expression

| Strain | *pmrA* locus | Concn of $Mg^{2+}$ in medium | Amt of alkaline phosphatase (U) produced[a] |
|---|---|---|---|
| CS283 | Wild type | 10 mM | 26.4 ± 6.5 |
| | Wild type | 10 μM | 424.9 ± 53.6 |
| KCS040 | *pmrA*::Tn*10d* | 10 μM | 388.5 ± 81.0 |

[a] The data shown are averages ± standard deviations of three independent cultures.

Zhou et al. showed that phosphoethanolamine-modified lipid A species are much less abundant than L-Ara4N-modified lipid A species in wild-type *S. enterica* serovar Typhimurium. Phosphoethanolamine-modified lipid A accumulated to high levels in *pmrA*-constitutive strains that harbored a null mutation in either *pmrE* or *pmrF* (44). Recently, it was shown that the *pmrA*-regulated *pmrC* gene mediates the phosphoethanolamine modification of lipid A (22). Although the possibility exists that these results are related to the substrate availability (since phosphoethanolamine and L-Ara4N modify the same site in lipid A), this finding suggests the possibility that L-Ara4N modification also interferes with phosphoethanolamine modification through a posttranslational effect.

We did not detect phosphoethanolamine-modified lipid A species in a *pmrE* or *pmrF* mutant strain cultivated in $Mg^{2+}$-limited medium upon analysis by MALDI-TOF mass spectrometry. These results were consistent with previous results that *m/z* peaks representing phosphoethanolamine-modified lipid A species were not observed in lipid A prepared from *pmrA*-constitutive strains that harbored a null mutation in either *pmrE* or *pmrF* (11). Since it is plausible that our inability to detect phosphoethanolamine-modified lipid A species by MALDI-TOF mass spectrometry was a result of a purification or analysis artifact, further purification by DEAE-cellulose and thin-layer chromatography, which was adopted by Zhou et al. (44), might be essential for the detection of phosphoethanolamine-modified lipid A species. Therefore, it is possible that phosphoethanolamine-containing lipid A also inhibits PagL activity. However, since there is no evidence that PmrE is essential for the addition of phosphoethanolamine to lipid A, it is likely that the presence or absence of this modification is less important for PagL inhibition than the L-Ara4N modification.

The L-Ara4N modification of lipid A increases bacterial resistance to cationic antimicrobial peptides, including polymyxin (11). In contrast, the 3-*O*-deacylation of lipid A reduces its potential to be recognized by human Toll-like receptor 4, which elicits host innate immune responses (18). Therefore, both lipid A modifications may be beneficial for bacterial survival in host tissues. Since both regulatory systems have periplasmic sensing domains, it is possible that conditions can exist in which PmrA-PmrB is repressed and PhoP-PhoQ is activated, although such in vitro growth conditions are currently unknown. PmrA-PmrB has been shown to be important in the intestinal environment of mice, as it is essential for maximal pathogenesis for mice by the oral route (12), whereas

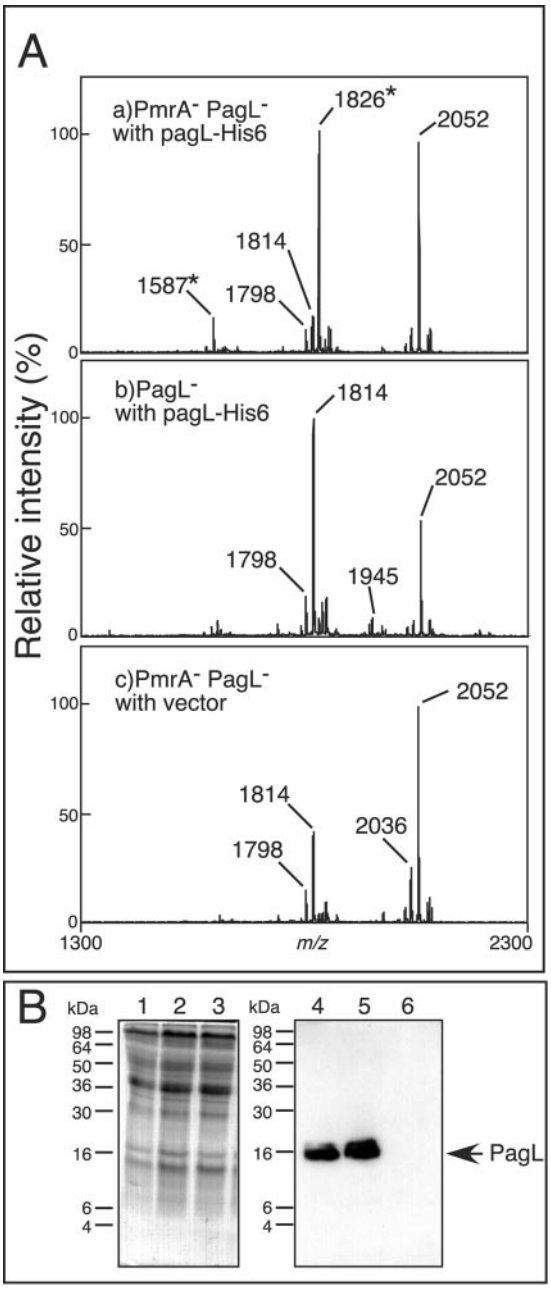


FIG. 4. Expression of recombinant PagL in a *pmrA*-null mutant strain, but not in a wild-type *pmrA* strain, induced lipid A deacylation. (A) Lipid A species prepared from KCS040 transformed with pWKS30-*pagL*-His6 (a), CS283 transformed with pWKS30-*pagL*-His6 (b), and KCS040 transformed with pWKS30 (c) were analyzed by use of a Bruker BiflexIII mass spectrometer. Transformants were cultivated in growth medium containing 10 μM $MgCl_2$ and 10 μg of ampicillin/ml. *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are marked with asterisks. Structural interpretations of lipid A species are summarized in Table 2. (B) Fifty-microgram samples of membrane proteins prepared from strains cultivated as described for panel A were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by staining (lanes 1 to 3) or by Western blotting (lanes 4 to 6). Lanes 1 and 4, KCS040 transformed with pWKS30-*pagL*-His6; lanes 2 and 5, CS283 transformed with pWKS30-*pagL*-His6; lanes 3 and 6, KCS040 transformed with pWKS30.

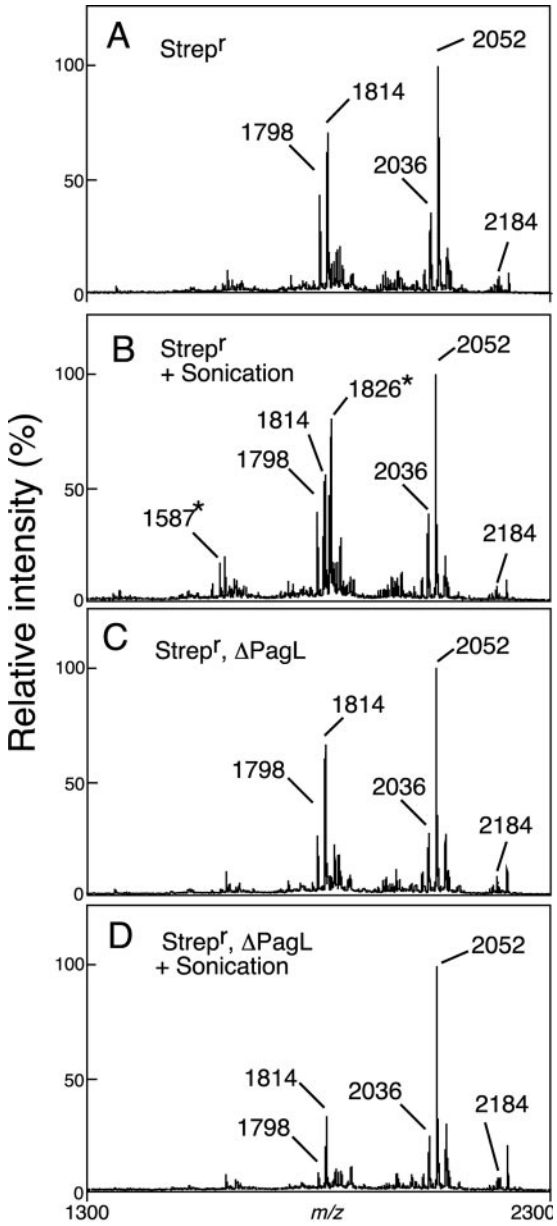


FIG. 5. Sonic disruption of *Salmonella* membranes promotes PagL-dependent lipid A deacylation. Strains CS401 and CS586 were cultivated in growth medium containing 10 μM $MgCl_2$. Cells collected from 25-ml cultures were suspended in 200 μl of 50 mM HEPES containing 250 mM NaCl, pH 7.5. Disruptions of cells were performed with a Virsonic 450 sonicator. Sonically disrupted and intact cells were incubated at 37°C for 1 h, and their lipid A species were analyzed by use of a Bruker BiflexIII mass spectrometer. (A) Intact CS401; (B) sonically disrupted CS401; (C) intact CS586; (D) sonically disrupted CS586. *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are marked with asterisks. Structural interpretations of lipid A species are summarized in Table 2.

PhoP-PhoQ is essential for systemic infections (26). Therefore, it is plausible that an environment in which PhoP-PhoQ is activated and PmrA-PmrB is repressed exists within host tissues.

A possible physiologic explanation for our observed results is that PagL is present in the membrane to react rapidly to alter

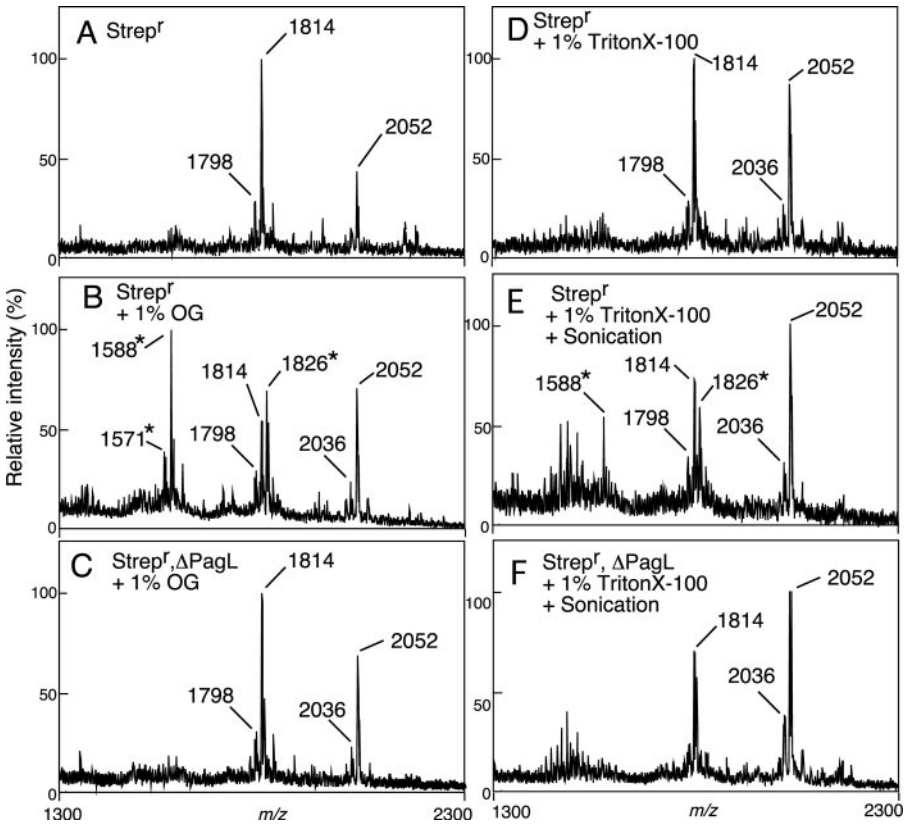


FIG. 6. Treatment of *Salmonella* membranes with *n*-octyl-β-D-glucopyranoside promotes PagL-dependent lipid A deacylation. Strains CS401 and CS586 were cultivated in growth medium containing 10 μM $MgCl_2$. Collected cells were treated with *n*-octyl-β-D-glucopyranoside or Triton X-100 at a final concentration of 1%. Additionally, sonic disruptions of Triton X-100-treated cells were performed. The cells were incubated at 37°C for 1 h, and their lipid A species were analyzed by use of a Voyager-DE STR mass spectrometer. (A) Intact CS401; (B) 1% *n*-octyl-β-D-glucopyranoside (OG)-treated CS401; (C) 1% *n*-octyl-β-D-glucopyranoside-treated CS586; (D) 1% Triton X-100-treated CS401; (E) 1% Triton X-100-treated and sonically disrupted CS401; (F) 1% Triton X-100-treated and sonically disrupted CS586. *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are marked with asterisks. Structural interpretations of lipid A species are summarized in Table 2.

lipid A in response to a specific type of membrane damage caused by a mammalian molecule with a similar activity to that of *n*-octyl-β-D-glucopyranoside. PmrA-PmrB-regulated outer membrane modifications, including the L-Ara4N modification of lipid A, might functionally repress lipid A deacylation by PagL, and the interaction of such molecules with the surface may release PagL from repression, with resultant lipid A deacylation.

Recently, Jia et al. reported that a brief EDTA treatment of *E. coli* induced lipid A palmitoylation by the outer membrane enzyme PagP (17). This result suggested that the migration of phospholipids into the outer leaflet can activate PagP enzymatic activity. However, PagP is also activated in the absence of any stimulation and under most growth conditions, as palmitoylation of lipid A was observed in *Salmonella* cells cultured in Luria broth (15). Interestingly, we did not observe PagL activity upon EDTA treatment, which most likely indicates that the simple promotion of phospholipid migration into the outer leaflet is not a signal for PagL activation. In contrast, a treatment with the detergent *n*-octyl-β-D-glucopyranoside, but not Triton X-100, released PagL from inhibition by L-Ara4N-containing membranes. Although both *n*-octyl-β-D-glucopyranoside and Triton X-100 are nonionic detergents, their structures are distinctly different. This structural difference may result in a physiological difference in membrane perturbation, such as the release of lipid A from the membrane, an alteration in membrane fluidity, or a change in the domain structure of PagL-interacting LPS. These differences might be responsible for the ability of detergents to induce lipid A deacylation by PagL, but the molecular machinery that enables or represses the ability of PagL to catalyze the removal of 3-hydroxymyristate from lipid A remains to be elucidated. Lipid A deacylation might be beneficial for the survival of salmonellae in host tissues, as gram-negative bacteria can continue to replicate, repairing and regenerating their outer membranes after damage. In this regard, released deacylated lipid A species that are less recognized by human Toll-like receptor 4 may provide a competitive advantage in host tissues.

## ACKNOWLEDGMENTS

We thank Martin Bader of our laboratory for his comments on the manuscript.

This work was supported by National Institutes of Health grant AI-30479 to S.I.M. and by a grant-in-aid for scientific research from the Japan Society for the Promotion of Science to K.K.

## REFERENCES

1. **Bader, M. W., W. W. Navarre, W. Shiau, H. Nikaido, J. G. Frye, M. McClelland, F. C. Fang, and S. I. Miller.** 2003. Regulation of *Salmonella typhimurium* virulence gene expression by cationic antimicrobial peptides. Mol. Microbiol. **50:**219–230.

2. **Belden, W. J., and S. I. Miller.** 1994. Further characterization of the PhoP regulon: identification of new PhoP-activated virulence loci. Infect. Immun. **62:**5095–5101.
3. **Bishop, R. E., H. S. Gibbons, T. Guina, M. S. Trent, S. I. Miller, and C. R. H. Raetz.** 2000. Transfer of palmitate from phospholipids to lipid A in outer membranes of gram-negative bacteria. EMBO J. **19:**5071–5080.
4. **Davis, R. W., D. Bolstain, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Ernst, R. K., T. Guina, and S. I. Miller.** 2001. *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. Microbes Infect. **3:**1327–1334.
6. **Ernst, R. K., E. C. Yi, L. Guo, K. B. Lim, J. L. Burns, M. Hackett, and S. I. Miller.** 1999. Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa.* Science **286:**1561–1565.
7. **Garcia Vescovi, E., F. C. Soncini, and E. A. Groisman.** 1996. $Mg^{2+}$ as an extracellular signal: environmental regulation of Salmonella virulence. Cell **84:**165–174.
8. **Gibbons, H. S., S. Lin, R. J. Cotter, and C. R. H. Raetz.** 2000. Oxygen requirement for the biosynthesis of the *S*-2-hydroxymyristate moiety in *Salmonella typhimurium* lipid A. J. Biol. Chem. **275:**32940–32949.
9. **Groisman, E. A.** 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. **183:**1835–1842.
10. **Groisman, E. A., E. Chiao, C. J. Lipps, and F. Heffron.** 1989. *Salmonella typhimurium phoP* virulence gene is a transcriptional regulator. Proc. Natl. Acad. Sci. USA **86:**7077–7081.
11. **Gunn, J. S., K. B. Lim, J. Krueger, K. Kim, L. Guo, M. Hackett, and S. I. Miller.** 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. **27:**1171–1182.
12. **Gunn, J. S., S. S. Ryan, J. C. Van Velkinburgh, R. K. Ernst, and S. I. Miller.** 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar Typhimurium. Infect. Immun. **68:**6139–6146.
13. **Gunn, J. S., and S. I. Miller.** 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178:**6857–6864.
14. **Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller.** 1997. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ.* Science **276:**250–253.
15. **Guo, L., K. B. Lim, C. M. Poduje, M. Daniel, J. S. Gunn, M. Hackett, and S. I. Miller.** 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptide. Cell **95:**189–198.
16. **Helander, I. M., I. Kilpelainen, and M. Vaara.** 1994. Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a $^{31}$P-NMR study. Mol. Microbiol. **11:**481–487.
17. **Jia, W., A. E. Zoeiby, T. N. Petruzziello, B. Jayabalasingham, S. Seyedirashti, and R. E. Bishop.** 2004. Lipid trafficking controls endotoxin acylation in outer membranes of *Escherichia coli.* J. Biol. Chem. **274:**44966–44975.
18. **Kawasaki, K., R. K. Ernst, and S. I. Miller.** 2004. 3-*O*-Deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of *Salmonella typhimurium*, modulates signaling through Toll-like receptor 4. J. Biol. Chem. **279:**20044–20048.
19. **Kier, L. D., R. M. Weppelman, and B. N. Ames.** 1979. Regulation of non-specific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. J. Bacteriol. **138:**155–161.
20. **Kox, L. F. F., M. M. S. M. Wosten, and E. A. Groisman.** 2000. A small protein that mediates the activation of a two-component system by another two-component system. EMBO J. **19:**1861–1872.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
22. **Lee, H., F. F. Hsu, J. Turk, and E. A. Groisman.** 2004. The PmrA-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica.* J. Bacteriol. **186:**4124–4133.
23. **Leive, L.** 1965. Release of lipopolysaccharide by EDTA treatment of *E. coli.* Biochem. Biophys. Res. Commun. **21:**290–296.
24. **Leive, L.** 1974. The barrier function of the gram-negative envelope. Ann. N. Y. Acad. Sci. **235:**109–127.
25. **Low, K. B., M. Ittensohn, T. Le, J. Platt, S. Sodi, M. Amoss, O. Ash, E. Carmichael, A. Chakraborty, J. Fischer, S. L. Lin, X. Luo, S. I. Miller, L. Zheng, I. King, J. M. Pawelek, and D. Bermudes.** 1999. Lipid A mutant *Salmonella* with suppressed virulence and TNFα induction retain tumor-targeting in vivo. Nat. Biotechnol. **17:**37–41.
26. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
27. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172:**2485–2490.
28. **Nelson, D. L., and E. P. Kennedy.** 1971. Magnesium transport in *Escherichia coli.* Inhibition by cobaltous ion. J. Biol. Chem. **246:**3042–3049.
29. **Nikaido, H.** 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. **67:**593–656.
30. **Nikaido, H., and M. Vaara.** 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49:**1–32.
31. **O'Brien, A. D., D. L. Rosenstreich, I. Scher, G. H. Campbell, R. P. MacDermott, and S. B. Formal.** 1980. Genetic control of susceptibility to *Salmonella typhimurium* in mice: role of the LPS gene. J. Immunol. **124:**20–24.
32. **Poltorak, A., X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoil, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. Science **282:**2085–2088.
33. **Raetz, C. R. H., and C. Whitfield.** 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. **71:**635–700.
34. **Rosner, M. R., J. Tang, I. Barzilay, and H. G. Khorana.** 1979. Structure of the lipopolysaccharide from an *Escherichia coli* heptose-less mutant. I. Chemical degradations and identification of products. J. Biol. Chem. **254:**5906–5917.
35. **Royle, M. C., S. Totemeyer, L. C. Alldridge, D. J. Maskell, and C. E. Bryant.** 2003. Stimulation of Toll-like receptor 4 by lipopolysaccharide during cellular invasion by live *Salmonella typhimurium* is a critical but not exclusive event leading to macrophage responses. J. Immunol. **170:**5445–5454.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Scherer, C. A., and S. I. Miller.** 2001. Molecular pathogenesis of salmonellae, p. 265–333. *In* E. A. Groisman (ed.), Principles of bacterial pathogenesis. Academic Press, San Diego, Calif.
38. **Somerville, J. E., Jr., L. Cassiano, B. Bainbridge, M. D. Cunningham, and R. P. Darveau.** 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. J. Clin. Investig. **97:**359–365.
39. **Trent, M. S., W. Pabich, C. R. H. Raetz, and S. I. Miller.** 2001. A PhoP/PhoQ-induced lipase (PagL) that catalyzes 3-*O*-deacylation of lipid A precursors in membranes of *Salmonella typhimurium.* J. Biol. Chem. **276:**9083–9092.
40. **Trent, M. S., A. A. Ribeiro, S. Lin, R. J. Cotter, and C. R. H. Raetz.** 2001. An inner membrane enzyme in *Salmonella* and *Escherichia coli* that transfers 4-amino-4-deoxy-L-arabinose to lipid A. J. Biol. Chem. **276:**43122–43131.
41. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli.* Gene **100:**195–199.
42. **Yi, E. C., and M. Hackett.** 2000. Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. Analyst **125:**651–656.
43. **Zhou, Z., S. Lin, R. J. Cotter, and C. R. H. Raetz.** 1999. Lipid A modifications characteristic of *Salmonella typhimurium* are induced by $NH_4VO_3$ in *Escherichia coli* K12. J. Biol. Chem. **274:**18503–18514.
44. **Zhou, Z., A. A. Ribeiro, S. Lin, R. J. Cotter, S. I. Miller, and C. R. H. Raetz.** 2001. Lipid A modifications in polymyxin-resistant *Salmonella typhimurium.* J. Biol. Chem. **276:**43111–43121.