

3-O-Deacylation of Lipid A by PagL, a PhoP/PhoQ-regulated Deacylase of *Salmonella typhimurium*, Modulates Signaling through Toll-like Receptor 4*

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Toll-like receptor 4 (TLR4)-mediated responses, which are induced by the lipid A portion of lipopolysaccharide, are important for host defense against *Salmonellae* infection. A variety of different data indicate that the acylation state of lipid A can alter TLR4-mediated responses. The *S. typhimurium* virulence gene product PhoP/PhoQ signals the presence of host microenvironments to regulate the expression of a lipid A 3-O-deacylase, PagL, and a lipid A palmitoyltransferase, PagP. We now demonstrate that 3-O-deacylation and palmitoylation of lipid A decreases its ability to induce TLR4-mediated signaling. Deacylated lipid A, deacylated and palmitoylated lipid A, palmitoylated lipid A, and unmodified lipid A species were purified from *Escherichia coli* heterologously expressing PagL and/or PagP. The purified lipid A preparations showed spectra of a single lipid A species on mass spectrometry and gave a single band on thin layer chromatography. The activity of purified lipid A species was examined using human and mouse cell lines that express recombinant human TLR4. Compared with unmodified lipid A, the modified lipid A species are 30–100-fold less active in the ability to induce NF- κ B-dependent reporter activation. These results suggest that the lipid A modifications reduce TLR4-signaling as part of *Salmonellae* adaptation to host environments.

Innate immune responses, such as secretion of cytokines by macrophages, are induced by an array of microbial components including lipopolysaccharide (LPS)¹ (1). LPS-hyporesponsive mutant mice, C3H/HeJ, are highly susceptible to infection by Gram-negative bacteria such as *Salmonella typhimurium* (2) and *Escherichia coli* (3), suggesting the importance of LPS-induced responses for host defense against bacterial infection. It has been shown that a point mutation in the cytoplasmic portion of Toll-like receptor 4 (TLR4) is responsible for the LPS hyporesponsiveness of C3H/HeJ mice (4, 5). Involvement of TLR4 in LPS-induced signaling was unequivocally demon-

strated by the generation of TLR4-deficient mice (6). In addition, MD-2, a molecule that physically associates with extracellular portion of TLR4, has been shown to be essential for TLR4-dependent LPS signaling (7, 8). The TLR4-MD-2 complex directly interacts with LPS (9, 10), especially with its hydrophobic membrane anchor portion of lipid A (9), suggesting that the TLR4-MD-2 complex is an essential component for LPS recognition.

Lipid A is the bioactive component of LPS that is also known as endotoxin (11). The structure of lipid A is relatively conserved among different pathogenic bacteria. Lipid A of *E. coli* and *S. typhimurium* is a β ,1'-6-linked disaccharide of glucosamine phosphorylated at the 1 and 4' positions and acylated at 2, 3, 2', and 3' positions with *R*-3-hydroxymyristate. The OH groups of the *R*-3-hydroxymyristate chains that are attached at positions 2' and 3' are further acylated with laurate and myristate, respectively (Fig. 1) (11).

S. typhimurium synthesizes enzymes in response to environmental conditions that perform covalent modifications of lipid A (11). Changes in lipid A acylation require the two component regulatory system PhoP/PhoQ (12), which is essential for *Salmonellae* virulence for mice (13) and humans (14). PhoQ is a sensor histidine kinase that responds to environmental conditions, which include those within mammalian tissues, such as macrophage phagosomes and those that destabilize the bacterial membrane such as magnesium-limited growth medium and exposure to antimicrobial peptides (15, 16). In response to specific environmental signals, PhoQ phosphorylates PhoP, leading to the activation or repression of over 40 different genes (15) including the genes involved in lipid A modifications such as the PhoP-activated gene *pagL* (17) and *pagP* (18, 19). *PagP* encodes a palmitoyltransferase that is located in the bacterial outer membrane (19). It transfers palmitate from glycerophospholipids in the inner leaflet of the outer membrane to the outer leaflet, which is almost exclusively composed of lipid A. This remodeling of the outer membrane increases bacterial resistance against antimicrobial peptides (18). Previously, when a heterogeneous mixture of modified LPS species from *S. typhimurium* was analyzed for proinflammatory responses, a reduction in LPS-induced cytokine production was observed in human umbilical cord endothelial cells (12). This finding suggested that bacterial cell surface remodeling upon infection of a host, *i.e.* specific LPS modification, might benefit the bacteria by reducing host innate immune responses. Consistent with this possibility, synthetic palmitoylated lipid A (P-lipid A) had reduced signaling in human macrophage-like cell lines (20).

PagL has been shown to encode lipid A 3-O-deacylase located in the outer membrane, but the biological function of the 3-O-deacylation is unknown (17). *Salmonellae* are intracellular pathogens, and they are unique among enteric bacteria in

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¹ The abbreviations used are: LPS, lipopolysaccharide; TLR, Toll-like receptor; TLC, thin layer chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; HEK, human embryonic kidney; U, unmodified; D, deacylated; P, palmitoylated; DP, deacylated and palmitoylated; luc, luciferase; Pag or *pag*, PhoP-activated gene.

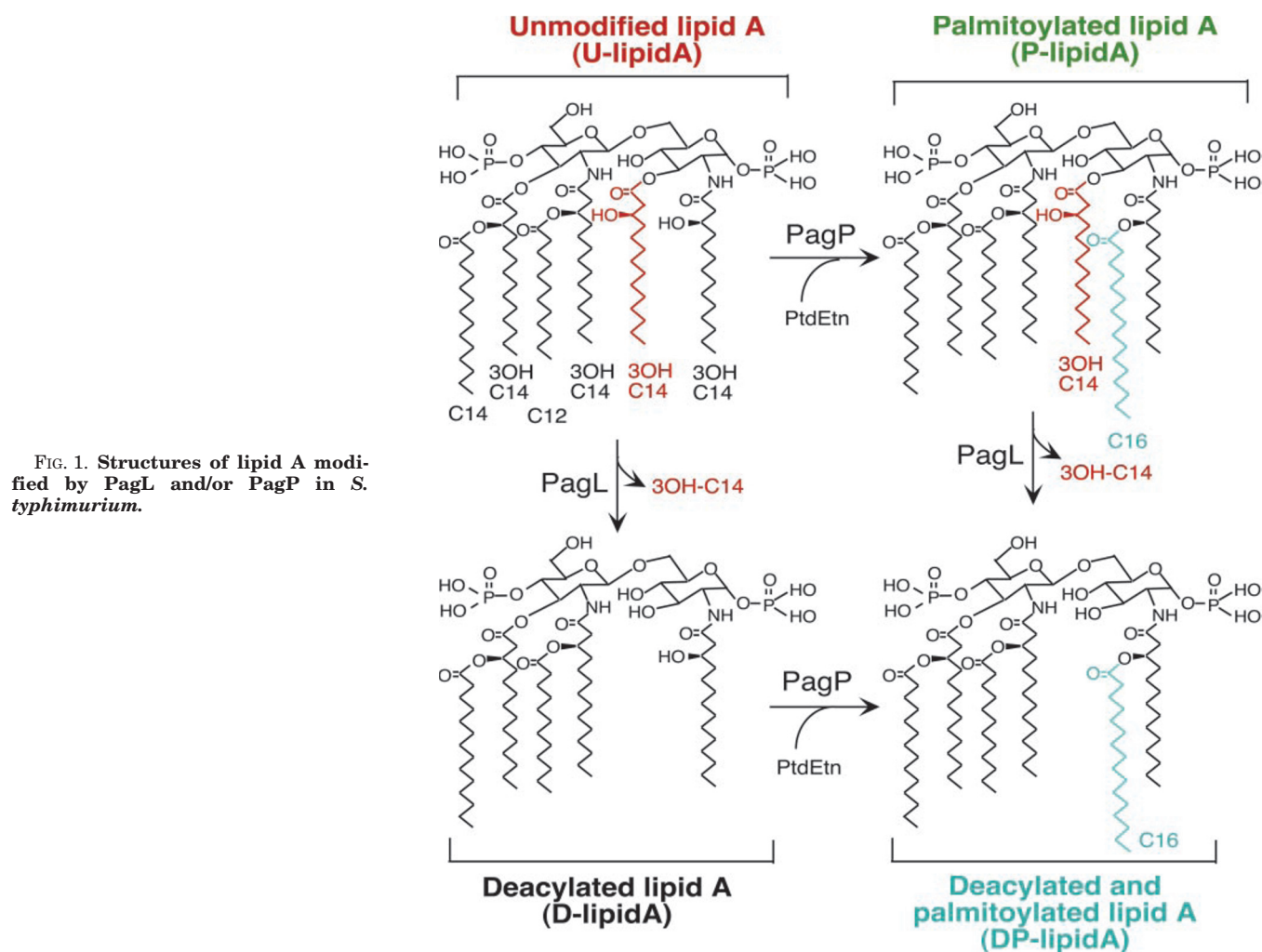


FIG. 1. Structures of lipid A modified by PagL and/or PagP in *S. typhimurium*.

having *pagL* in its genome, suggesting a specific function of PagL in *Salmonellae* pathogenesis. A possible function of *pagL* is modulation of lipid A recognition by the TLR4-MD-2 complex. To address this issue, we purified 3-*O*-deacylated lipid A species and show here that 3-*O*-deacylation of lipid A decreases its activity to induce cellular signaling through TLR4.

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals were reagent grade or better. Restriction endonucleases and DNA modifying enzymes were from New England Biolabs (Beverly, MA) and Invitrogen. Thin layer chromatography (TLC) Silica Gel 60 and HPTLC plates were from EM Science (Gibbstown, NJ). Oligonucleotides were prepared commercially by Invitrogen. QiaPrep spin miniprep kit and QiaQuick gel extraction kit were from Qiagen. *p*-Anisardehyde was from Sigma.

Plasmids—The *PagL* coding region was amplified from pWLP21 (17) by PCR with *Pfu* Turbo (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Primers used for the PCR were *PagL*-NcoI (5'-ATCATGCCATGGGCATGAAGAGAATATTTATATAT-3') and *PagL*-C-term (5'-CGCGGATCCTCAGAAATTATACTAATTGA-3'). The *PagP* coding region was amplified from pLG18 (18) by PCR with primers *PagP*-N-term (5'-TCAATTAGTTATAATTTCTGAAGGAGGAATTCACCATGATCATCAGAAAGTATTTTCTT-3') and *PagP*-C-PstI (5'-GA-CTGCAGTCAAACTGGAACGCATCCAG-3'). The amplified DNA fragments containing *PagL* or *PagP* were linked by PCR with primers *PagL*-N-KpnI (5'-CCATGGTACCCATGAAGAGAATATTTATATAT-3') and *PagP*-C-PstI. The linked reaction product was cloned into KpnI/PstI sites of pBAD24 (21), and the resulting expression construct was named pBAD24-*PagL*-*PagP*. The *PagP* coding region was amplified from pLG18 (18) by PCR with primers *PagP*-N-KpnI (5'-CCATGGTACCCATGATCATCAGAAAGTATTTTCTT-3') and *PagP*-C-PstI. The reaction product was cloned into KpnI/PstI site of pBAD24 (21) under control of the arabinose P_{BAD} promoter, and the resulting expression construct

was named pBAD24-*PagP*. The inserts of the expression constructs were verified by sequencing at the biochemistry DNA sequencing facility at University of Washington.

Purification of Lipid A Species—For the preparation of deacylated lipid A (D-lipid A) and deacylated and palmitoylated lipid A (DP-lipid A) species (Fig. 1), *E. coli* strain XL1-Blue (Stratagene) was transformed with pBAD24-*PagL*-*PagP*. The transformant was cultivated at 37 °C in LB medium containing 100 µg/ml ampicillin until $A_{600} = \sim 0.5$, and then expressions of recombinant *PagL* and *PagP* were induced by the addition of L-(+)-arabinose (2 mg/ml). After cultivation for 4 h in the presence of arabinose, cells were collected. Preparation of lipid A was performed as described previously (22). The prepared lipid A was dissolved in chloroform/methanol (4:1, v/v) and applied in a line to Silica Gel 60 TLC plates (EM Science, Gibbstown, NJ). The plates were developed in *n*-propanol/water/28% ammonium hydroxide (75:5:25, v/v). The bands containing lipid A species were transiently visualized by spraying water, and then a band ($R_f = \sim 0.22$) that contains both D-lipid A and DP-lipid A species was scraped off of the plates. Extraction of lipid A species from silica gel was performed as described previously (22). Extracted lipid A species were dissolved in chloroform/methanol (4:1, v/v) and applied in a line to Silica Gel 60 TLC plates. The plates were developed in chloroform/pyridine/88% formic acid/methanol/water (60:35:10:5:2, v/v), which was previously used for analysis of lipid A species (23). The bands containing D-lipid A or DP-lipid A species were extracted from silica gel as described above. Identification of lipid A species was performed by mass spectrometry (see below).

For the preparation of U-lipid A and P-lipid A (Fig. 1), XL1-Blue strain was transformed with pBAD24-*PagP*. Induction of recombinant *PagP* and preparation of lipid A were performed as described above. The prepared lipid A was dissolved in chloroform/methanol (4:1, v/v) and applied in a line to Silica Gel 60 TLC plates. The plates were developed in chloroform/pyridine/88% formic acid/methanol/water (60:35:10:5:2, v/v). The bands containing lipid A species were extracted from silica gel as described above.

Quantification of lipid A species was performed by measuring the amount of phosphate as described previously (24) with slight modifications. 140 μ l of 70% perchloric acid were added to dried samples. After incubation at 150 $^{\circ}$ C for 1 h, 800 μ l of water/1.25% ammonium molybdate/10% ascorbic acid (5:2:1, v/v/v) was added to the sample mixture and incubate at 100 $^{\circ}$ C for 5 min. Absorbance at 820 nm then was measured. Potassium phosphate was used as standard for quantifica-

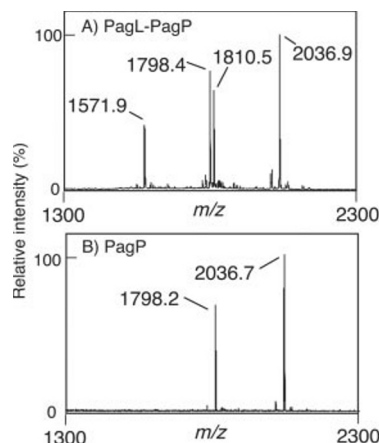


FIG. 2. Generation of 3-O-deacylated and/or palmitoylated lipid A species by heterologous expression of recombinant PagL and/or PagP in *E. coli*. Expressions of recombinant PagL and/or PagP protein are induced in *E. coli* XL-1-Blue strains transformed with pBAD24-PagL-PagP (A) or pBAD24-PagP (B) as described under "Experimental Procedures." Lipid A was isolated as described previously (33) and analyzed by MALDI-TOF MS.

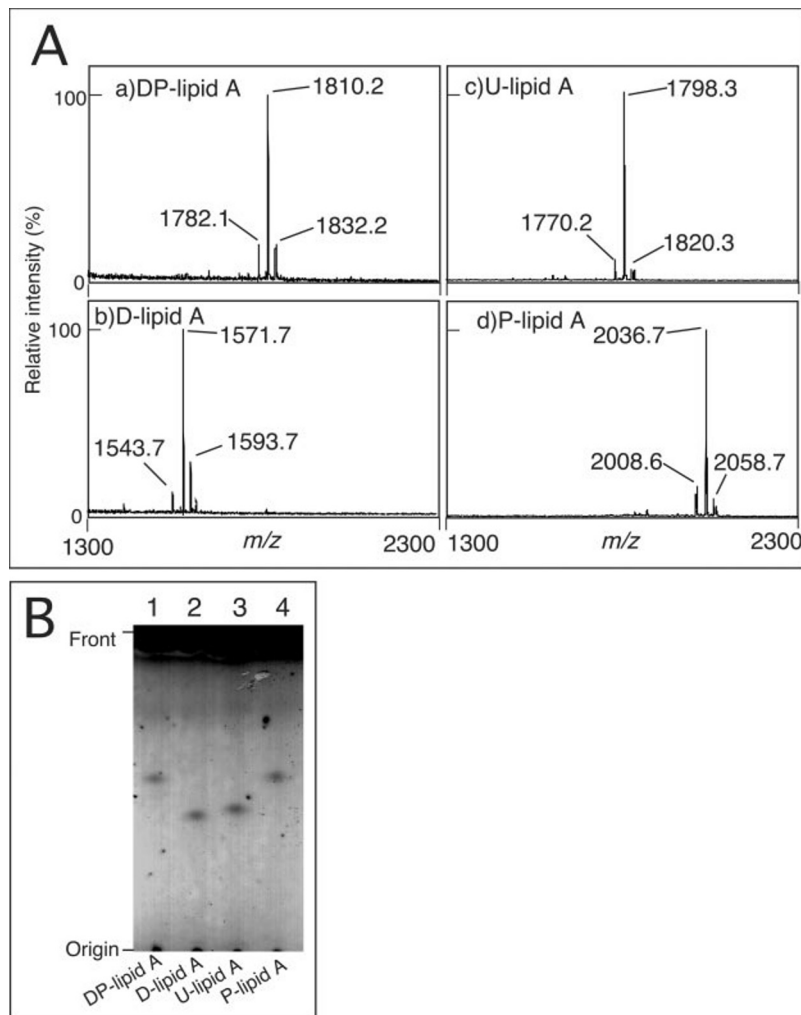
tion. From a 2,000-ml culture of *E. coli* transformed with pBAD24-PagL-PagP, 16.6 μ g of D-lipid A and 15.7 μ g of DP-lipid A species were purified. From a 100-ml culture of *E. coli* transformed with pBAD24-PagP, 10.8 μ g of U-lipid A and 23.6 μ g of P-lipid A species were purified.

Mass Spectrometry (MS)—Samples were dissolved in 20 mg/ml 5-chloro-2-mercaptobenzothiazole matrixes in chloroform/methanol (1:1, v/v). Spectra were obtained in the negative reflection mode using a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) Bruker BiflexIII mass spectrometer (Bruker Daltonics, Inc., Billerica, MA). Each spectrum was the average of 200 shots. Lipid A from *S. typhimurium* Re-mutant (Ribi Immunochem Research, Inc., Hamilton, MT) was used as an external mass calibrant.

Mammalian Cell Lines and Cell Culture—A mouse pro-B cell line, Ba/F3, stably expressing epitope-tagged human TLR4, epitope-tagged human MD-2, and p55Ig κ luc, a NF- κ B-dependent luciferase reporter construct, was a generous gift from Dr. K. Miyake (7) and was named Ba/hTLR4/hMD2 (25). A Ba/F3 cell line introduced with p55Ig κ luc was established previously (26) and was named Ba/luc here. Ba/F3 cell lines were maintained as described previously (25). pEFBOS (27) vector-based expression constructs, which encode human TLR4 or human MD-2 cDNA bearing a FLAG tag followed by His₆ tag at the C terminus, were generous gifts from Dr. K. Miyake (28). The expression constructs and p55Ig κ luc (29) were introduced into human embryonic kidney (HEK) 293 cell line. The transformant was purified by limited dilution and was named 293/hTLR4/hMD2. 293/luc, a HEK293 cell line introduced with p55Ig κ luc, was established previously (30). HEK293 cell lines are maintained as described previously (30).

Luciferase Assay—100 μ l of cell culture in a well of 96-well dish (Costar, Cambridge, MA) were lysed by the addition of 25 μ l of 5 \times cell culture lysis reagent (Promega, Madison, WI). Luciferase activity was measured using 20 μ l of lysate and 100 μ l of luciferase assay substrate (Promega). The luminescence was quantified with a luminometer (Berthold Detection Systems, Oak Ridge, TN).

FIG. 3. Purification of U-lipid A, D-lipid A, P-lipid A, and DP-lipid A species. A, lipid A species purified from XL-1-Blue transformed with pBAD24-PagL-PagP (a and b) or pBAD24-PagP (c and d) were analyzed by MALDI-TOF MS. Major peaks at m/z 1810.2 (a), 1571.7 (b), 1798.3 (c), and 2036.7 (d) were interpreted as DP-, D-, U-, and P-lipid A species, respectively. B, 1 μ g of DP-lipid A (lane 1), D-lipid A (lane 2), U-lipid A (lane 3), and P-lipid A (lane 4) were spotted onto a Silica Gel 60 HPTLC plates. The plates were developed in chloroform/pyridine, 88% formic acid/methanol/water (60:35:10:5:2, v/v). The spots were visualized by heating the plate at 130 $^{\circ}$ C for ~60 min after spraying with a mixture of methanol/ H_2SO_4 /acetic acid/*p*-anisaldehyde (90:5:5:0.5, v/v).



RESULTS

Purification of 3-O-Deacylated and/or Palmitoylated Lipid A Species—Four species of differently acylated lipid A are generated by the modifications catalyzed by PagL and/or PagP (Fig. 1). We here named these species unmodified lipid A, deacylated lipid A, palmitoylated lipid A, deacylated and palmitoylated lipid A as U-lipid A, D-lipid A, P-lipid A, and DP-lipid A, respectively (Fig. 1). To generate and purify the lipid A species, a heterologous *E. coli* expression system was established. *E. coli* XL1-Blue strain was transformed with pBAD24-PagL-PagP, a PagL and PagP expression construct, or pBAD24-PagP, a PagP expression construct. Lipid A species of the strains were analyzed by MALDI-TOF MS. The negative-ion spectrum of lipid A prepared from *E. coli* transformed with pBAD24-PagL-PagP demonstrated major peaks at m/z 1571.9, 1798.4, 1810.5, and 2036.9 (Fig. 2A), and their structures were interpreted as D-lipid A, U-lipid A, DP-lipid A, and P-lipid A, respectively (Fig. 1) (17, 18). The negative-ion spectrum of lipid A prepared from *E. coli* transformed with pBAD24-PagP demonstrated major peaks at m/z 1798.2 and 2036.7 (Fig. 2B), and their structures were interpreted as U-lipid A and P-lipid A, respectively (Fig. 1) (18). These results show that the 3-O-deacylated and/or palmitoylated lipid A species are generated by the heterologous *E. coli* expression system.

D-lipid A and DP-lipid A species were prepared from *E. coli* transformed with pBAD24-PagL-PagP, and U-lipid A and P-lipid A species were prepared from *E. coli* transformed with pBAD-PagP as described under “Experimental Procedures.” The negative-ion spectrum of each lipid A preparation demonstrated a major peak at m/z 1810.2, 1571.7, 1798.3, and 2036.7, and their structures were interpreted as DP-lipid A, D-lipid A, U-lipid A, and P-lipid A, respectively (Fig. 3A). In addition, minor peaks, m/z values of which are +22 or –28 of that of the major peak (Fig. 3A), such as m/z 1832.2 and 1782.1 in DP-lipid A (m/z 1810.2) preparation were observed (Fig. 3Aa). The +22 peaks were interpreted as sodium adducts. To ensure that individual lipid A species were isolated, 1 μ g of the prepared lipid A species was analyzed by TLC under the solvent system that was used for the final purification step. Each lipid A preparation showed a single band with similar intensity (Fig. 3B). Taken together, these results indicate that each of the lipid A species, U-, D-, P-, and DP-lipid A, was purified.

3-O-Deacylated and/or Palmitoylated Lipid A Species Show Decreased Ability to Induce NF- κ B Activation through TLR4—To investigate the biological effect of 3-O-deacylation and/or palmitoylation of lipid A in *S. typhimurium*, we examined whether the modification(s) of lipid A changed its ability to induce NF- κ B activation through TLR4. Mouse pro-B cell line Ba/F3 and HEK293 cell lines, both of which confer LPS responsiveness by expressions of TLR4 and MD-2 (7, 30), were used for the analysis. Purified U-lipid A induced TLR4-MD-2-dependent NF- κ B activation on Ba/hTLR4/hMD2 and 293/hTLR4/hMD2 cells (Fig. 4A). Ba/hTLR4/hMD2 and 293/hTLR4/hMD2 cells then were stimulated with various concentrations of the purified lipid A species, U-, D-, P-, and DP-lipid A. The Ba/hTLR4/hMD2 cell line showed ~10-fold induced reporter activation on 0.1 ng/ml U-lipid A, 3 ng/ml P-lipid A, 3–10 ng/ml D-lipid A, or 10 ng/ml DP-lipid A stimulation (Fig. 4B), suggesting that modified lipid A species are from 30- to 100-fold less active in induction of Ba/hTLR4/hMD2 cell NF- κ B activation. To further confirm this result, 293/hTLR4/hMD2 cells were also tested. These cells show ~5–10-fold induced reporter activation on exposure to 10–30 ng/ml U-lipid A or 1,000 ng/ml of D-lipid A, P-lipid A, or DP-lipid A stimulation, indicating that modified lipid A species are from 30- to 100-fold less active in induction of 293/hTLR4/hMD2 cell NF- κ B activation. (Fig. 4C).

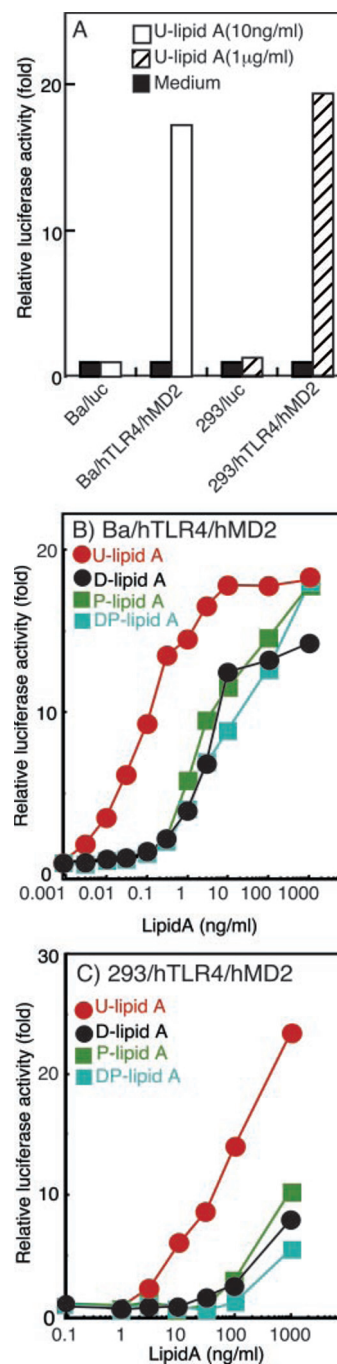


FIG. 4. 3-O-Deacylated and/or palmitoylated lipid A species show decreased ability to induce NF- κ B activation through TLR4. A, Ba/luc, Ba/hTLR4/hMD-2, 293/luc, and 293/hTLR4/hMD-2 cells were cultivated in culture medium (Medium) or in culture medium containing 10 ng/ml or 1 μ g/ml U-lipid A for 4 h, and then luciferase activity was measured. Luciferase activity was normalized as to the activity in the cells cultivated in culture medium and presented as fold induction. The column indicates the averages for triplicate wells. B and C, Ba/hTLR4/hMD-2 cells (B) and 293/hTLR4/hMD-2 cells (C) were cultivated for 4 h in culture medium containing the indicated amount of U-lipid A (red), D-lipid A (black), P-lipid A (green), or DP-lipid A (blue), and then luciferase activity was measured. Luciferase activity was presented as in A. Data represent the averages of duplicate wells, and the results shown are representative of two independent experiments.

DISCUSSION

In this paper, we purified lipid A species modified by the *S. typhimurium* PhoP/PhoQ-regulated outermembrane enzyme, PagL and/or PagP. Using the purified lipid A species, we demonstrated that Salmonellae lipid A 3-O-deacylation by PagL

and palmitoylation by PagP reduces the activity of lipid A in inducing cellular signaling through TLR4. These results are consistent with the results reported by Hajjar *et al.* (31) that indicate that lipid A species from *Pseudomonas aeruginosa*, which consistently express a 3-*O*-deacylase, are less active than hexa-acylated lipid A from *E. coli* for induction of signaling through human TLR4. Recently, using synthetic lipid A mimetics, Stover *et al.* (32) described that a hexa-acylated structure may be optimum for human TLR4 signaling, perhaps by adopting a conical-like structure that has been postulated to be associated with agonist activity. Our results are consistent with their results in that penta-acylated D-lipid A and hepta-acylated P-lipid A are less active than hexa-acylated U-lipid A. However, our results also indicate that a hexa-acylated DP-lipid A is less active than U-lipid A. These results suggest that TLR4-dependent lipid A signaling is not simply related to the number of fatty acids but also to their location and length on the glucosamine backbone.

This work also suggests that inactivation of lipid A by 3-*O*-deacylation and palmitoylation of lipid A to reduce TLR4-signaling could be an important strategy of pathogenic *Salmonellae*. Recent results with synthetic lipid A mimetics indicate that different structures can stimulate different gene expression profiles (32). In addition to the potential benefit to intracellular *Salmonellae* of reduction in TLR4-signaling, deacylation of lipid A could also result in an alteration of the pattern of host cell gene expression in infected cells. Therefore, such modifications of lipid A by PagL and/or PagP may enable *S. typhimurium* to escape maximum recognition by TLR4-MD-2 complex and alter host cell responses that could promote intracellular survival.

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