THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 12, Issue of March 23, pp. 9083–9092, 2001
Printed in U.S.A.

A PhoP/PhoQ-induced Lipase (PagL) That Catalyzes 3-*O*-Deacylation of Lipid A Precursors in Membranes of *Salmonella typhimurium**

Received for publication, November 28, 2000
Published, JBC Papers in Press, December 6, 2000, DOI 10.1074/jbc.M010730200

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Pathogenic bacteria modify the structure of the lipid A portion of their lipopolysaccharide in response to environmental changes. Some lipid A modifications are important for virulence and resistance to cationic antimicrobial peptides. The two-component system PhoP/PhoQ plays a central role in regulating lipid A modification. We now report the discovery of a PhoP/PhoQ-activated gene (*pagL*) in *Salmonella typhimurium*, encoding a deacylase that removes the *R*-3-hydroxymyristate moiety attached at position 3 of certain lipid A precursors. The deacylase gene (*pagL*) was identified by assaying for loss of deacylase activity in extracts of 14 random TnphoA::*pag* insertion mutants. The *pagL* gene encodes a protein of 185 amino acid residues unique to *S. typhimurium* and closely related organisms such as *Salmonella typhi*. Heterologous expression of *pagL* in *Escherichia coli* on plasmid pWLP21 results in loss of the *R*-3-hydroxymyristate moiety at position 3 in ~90% of the lipid A molecules but does not inhibit cell growth. PagL is synthesized with a 20-amino acid *N*-terminal signal peptide and is localized mainly in the outer membrane, as judged by assays of separated *S. typhimurium* membranes and by SDS-polyacrylamide gel analysis of membranes from *E. coli* cells that overexpress PagL. The function of PagL is unknown, given that *S. typhimurium* mutants lacking *pagL* display no obvious phenotypes, but PagL might nevertheless play a role in pathogenesis if it serves to modulate the cytokine response of an infected animal host.

Pathogenic bacteria are capable of sensing microenvironments within the tissues of their animal hosts, leading to the expression of virulence genes necessary for bacterial survival and replication (1, 2). In *Salmonella typhimurium* and *Salmonella typhi*, some virulence genes are controlled by the two-component regulatory system PhoP/PhoQ (3, 4). At low levels of Mg^{2+}, the PhoQ sensor protein phosphorylates and activates the transcriptional regulatory protein PhoP, which in turn either activates or represses over 40 different genetic loci (5, 6). A second two-component regulatory system, PmrA/PmrB, is itself PhoP/PhoQ-activated (5, 7). PmrA is also activated directly by the PmrB kinase in the presence of ferric ions or indirectly at low pH (8). Mutants altered in the PhoP/PhoQ system display greatly reduced virulence (9, 10). Homologues of both regulatory systems are present in other Gram-negative bacteria, including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Yersinia pestis* (4, 11).

Among their many functions, the PhoP/PhoQ and the PmrA/PmrB systems regulate the expression of gene products involved in the covalent modification of lipid A (12), the glycolipid anchor of lipopolysaccharide (LPS).[1] LPS is a major component of the outer leaflet of the outer membranes of Gram-negative bacteria, and the lipid A portion of LPS is the bioactive component that is also known as endotoxin (13–16). During bacterial infections of animals, lipid A activates the innate immune system through interaction with Toll-like receptors, primarily TLR-4 (17–20). The host response to lipid A includes the production of cationic antimicrobial peptides, cytokines, tissue factor, and additional immunostimulatory molecules (19–22). In limited infections, the response to lipid A helps to clear the bacteria, but in overwhelming sepsis, high levels of circulating cytokines and procoagulant activity may damage the microvasculature and precipitate the syndrome of Gram-negative septic shock with disseminated intravascular coagulation (23, 24).

The structure of lipid A is relatively conserved among different pathogenic Gram-negative bacteria (13, 14, 16, 25). 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 the 2-, 3-, 2′-, and 3′-positions with *R*-3-hydroxymyristate (Fig. 1) (13, 14, 16, 25). 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 (13, 14, 16, 25). Lipid A is glycosylated at position 6′ with two 3-deoxy-D-*manno*-octulosonic acid (Kdo) moieties (Fig. 1) (13, 14, 16, 25). Under certain circumstances, additional covalent modifications of lipid A are present, including 4-amino-4-deoxy-L-arabinose (4-aminoarabinose), phosphoethanolamine, palmitate, and/or 2-hydroxymyristate moieties (Fig. 1) (12, 26–28). In *S. typhimurium*, Miller and co-workers have shown that incorporation of palmitate and 2-OH myristate moieties (12, 29) is controlled by PhoP/PhoQ, whereas the 4-aminoarabinose and phosphoethanolamine modifications require the activation of PmrA (27, 30).

PhoP-PhoQ mutants are more sensitive to the action of certain cationic antimicrobial peptides, in part because of the loss of palmitoylation of lipid A in the absence of the function of the

* This research was supported by National Institutes of Health Grants GM-51310 (to C. R. H. R.) and AI-30479 (to S. I. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by National Institute of Health Grant 1 F32 AI1056-01.

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[1] The abbreviations used are: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-octulosonic acid; PCR, polymerase chain reaction; CAPS, 3-(cyclohexylamino)propanesulfonic acid; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

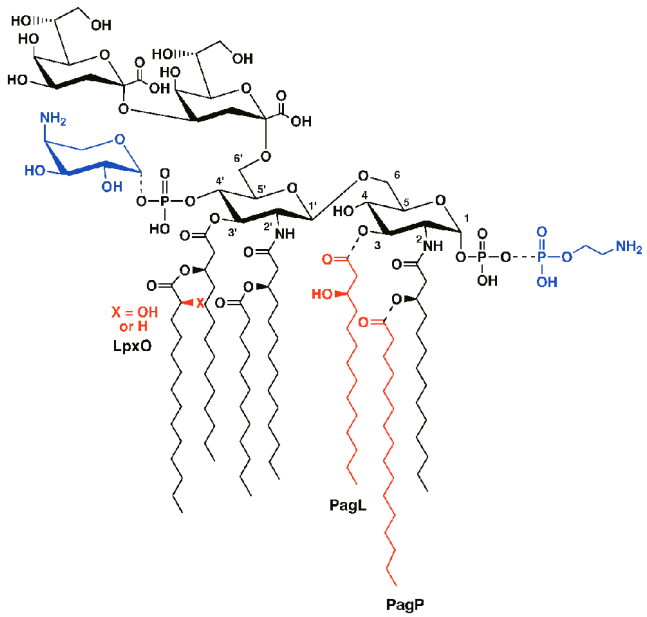

FIG. 1. **Regulated modifications of the structure of Kdo_2-lipid A in *S. typhimurium*.** The phosphate residues and acyl chains of lipid A in *S. typhimurium* can be derivatized in a regulated fashion (12). The phosphate moieties of lipid A can be substituted with 4-amino-4-deoxy-L-arabinose and/or phosphoethanolamine groups, both of which are under PmrA/B control (*blue* substituents) (26, 56). Minor species are present in which the locations of the 4-amino-4-deoxy-L-arabinose and phosphoethanolamine groups are reversed (57) (Z. Zhou and C. R. H. Raetz, manuscript in preparation) or in which both phosphates are modified with the same substituent (not shown). The addition of the palmitate chain is catalyzed by PagP, as indicated (31), and formation of the 2-hydroxymyristate group (*X*) requires a novel hydroxylase homologue, designated LpxO (58). The ester-linked β-hydroxymyristoyl chain at the 3-position may be removed by the outer membrane lipase PagL, as indicated. Substituents that are incorporated or removed in a PhoP/Q-dependent manner are shown in *red*.

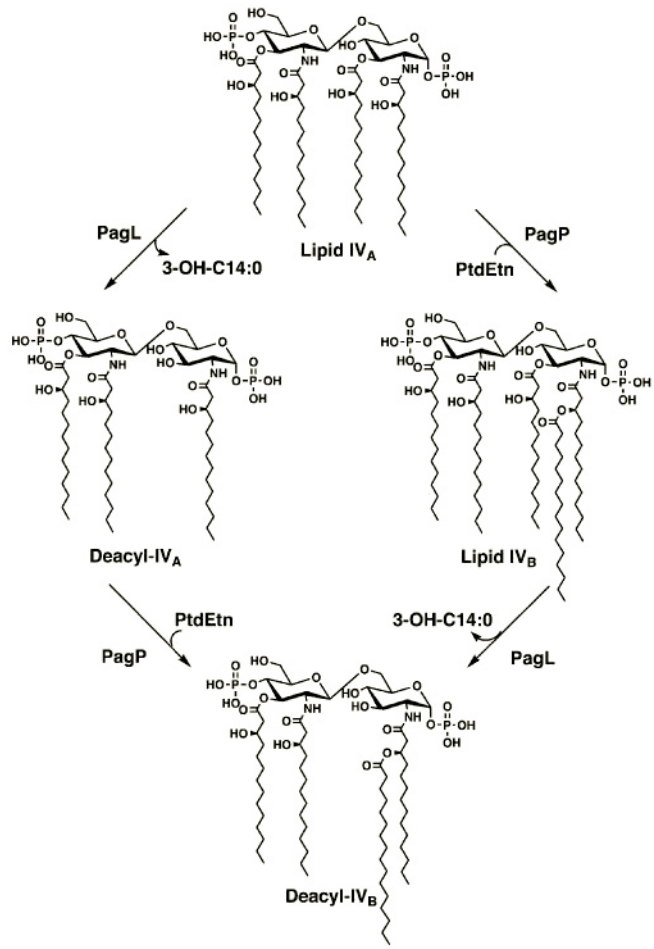

FIG. 2. **Reactions catalyzed by PagP and PagL.** These reactions may occur in either order when lipid IV_A is used as the substrate.

PhoP-activated gene *pagP* (29). We have recently shown that *pagP* is the structural gene for a novel acyltransferase (31) (Fig. 2) that utilizes glycerophospholipids as palmitate donors (31, 32). PagP is the first example of a lipid A biosynthetic enzyme localized to the outer membrane (31).

In the course of characterizing lipid A modifications in extracts of different *S. typhimurium* mutants, we have discovered a novel 3-*O*-deacylase activity that is strongly regulated by PhoP/PhoQ (Fig. 2). In the present study, we demonstrate that the 3-*O*-deacylase, like the PagP acyltransferase, is found mainly in the outer membrane. By assaying for 3-*O*-deacylase activity in extracts of PhoP-constitutive *S. typhimurium* strains harboring insertion mutations in different PhoP-activated (*pag*) genes (33), the structural gene (*pagL*) encoding the deacylase was identified. The *pagL* gene was sequenced and shown to be unique to strains of *Salmonella*. When expressed in *E. coli*, PagL activity is localized in the outer membrane, and extensive lipid A 3-*O*-deacylation occurs without loss of cell viability. The function of *pagL* is unknown, since nonpolar deletions of *S. typhimurium pagL* display no obvious phenotypes. However, partial 3-*O*-deacylation of *Salmonella* lipid A could be advantageous under certain conditions, since it might modulate the cytokine response of the host during an infection.

EXPERIMENTAL PROCEDURES

Chemicals and Other Materials—[γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences. Silica gel 60 (0.25-mm) thin layer plates were purchased from EM Separation Technologies. Tryptone and yeast extract were from Difco. Triton X-100 and bicinchoninic acid were from Pierce. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

Bacterial Strains—The bacterial strains used in the present study are described in Table I. Typically, bacteria were grown at 37 °C in LB medium, which consists of 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter (34). In experiments involving Mg^{2+} limitation or pH changes, cells were grown in N-minimal medium (35) with varying concentrations of Mg^{2+} at pH 7.7 in 100 mM Tris-HCl or at pH 5.8 in 100 mM bis-Tris buffer at 37 °C. Cells (10 ml) were first grown overnight at pH 7.7, harvested by centrifugation, washed twice with 5 ml of N-minimal medium at pH 7.7, and diluted 1:100 into N-minimal medium at pH 5.8 or 7.7, containing either low (10 μM) or high (10 mM) $MgCl_2$. Cells were then grown into late log phase at 37 °C and harvested at A_{600} ranging from 0.65 to 1.0. When appropriate, cultures were supplemented with 100 μg/ml ampicillin, 12 μg/ml tetracycline, 30 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

Preparation of Radiolabeled Substrates—The substrate [4′-^{32}P]lipid IV_A was prepared using 100 μCi of [γ^{32}-P]ATP, tetraacyl-disaccharide 1-phosphate acceptor and membranes from *E. coli* that overexpress the 4′-kinase, as previously described (36), with the following minor changes. After the 4′-kinase reaction was completed, the assay mixture was spotted onto a 10 × 20-cm TLC plate. The plate was dried under a cold air stream and developed in the solvent system chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v). Following chromatography, the plate was dried again and exposed to x-ray film for 30 s to locate the [4′-^{32}P]lipid IV_A. The region of the silica plate containing the product was removed by scraping, transferred to a thick walled glass tube, and resuspended in 3 ml of an acidic single-phase Bligh/Dyer mixture (37), consisting of chloroform/methanol/0.1 M HCl (1:2:0.8, v/v/v). The suspension was vigorously mixed with the aid of a vortex and subjected to sonic irradiation for 30 s. The silica particles were removed with a clinical centrifuge set at top speed for 10 min. The supernatant containing the ^{32}P labeled lipid was removed, and the extraction process was repeated. The extracted materials were pooled. The solution was then converted to a two-phase Bligh/Dyer mixture (37), consisting of chloroform/methanol/0.1 M HCl (2:2:1.8, v/v/v). The phases were separated in a clinical centrifuge, and the lower phase was removed to a separate tube. The resulting upper phase was extracted a second time

TABLE I
Relevant bacterial strains and plasmids

Strain/Plasmid	Description	Source or reference
S. typhimurium strains		
ATCC 14028s	Wild type	ATCC
CS022	*pho-24* (PhoP-constitutive)	Ref. 49
CS015	CSO22, *phoP102*::Tn10d-cam	Ref. 69
CS400	CSO22, *pagA*::Tn10d	Ref. 51
CS027	CSO22, *pagC1*::TnphoA	Ref. 51
CS336	CSO22, *pagD*::TnphoA	Ref. 51
CS325	CSO22, *pagE*::TnphoA	Ref. 51
CS332	CSO22, *pagF*::TnphoA	Ref. 51
CS324	CSO22, *pagG*::TnphoA	Ref. 51
CS331	CSO22, *pagH*::TnphoA	Ref. 51
CS334	CSO22, *pagI*::TnphoA	Ref. 51
CS327	CSO22, *pagK*::TnphoA	Ref. 51
CS328	CSO22, *pagL*::TnphoA	Ref. 51
CS333	CSO22, *pagM*::TnphoA	Ref. 51
CS326	CSO22, *pagN*::TnphoA	Ref. 51
CS329	CSO22, *pagO*::TnphoA	Ref. 51
CS330	CSO22, *pagP*::TnphoA	Ref. 51
CS019	*phoN2* zxx::6251 Tn10d-cam	Ref. 69
JSG435	*pmrA*505 zjd::Tn10d-cam (PmrA-constitutive)	Ref. 7
JSG421	*pmrA*::Tn10d	Ref. 7
CS401	$Strep^R$ CS019	S. I. Miller
CS491	$Strep^R$ CS022	S. I. Miller
CS584	CS491 Δ*pagL*	This work
CS586	CS401 Δ*pagL*	This work
E. coli strains		
W3110	Wild type, F^-, λ^-	*E. coli* Genetic Stock Center (Yale)
MC1061	*araD139*Δ(*ara-leu*)7697*hsdRhds*M^+	*E. coli* Genetic Stock Center (Yale)
XL-1 Blue-MR	Δ*mcrABC*, *recA*1, *lac*	Stratagene
BLR(DE3)pLysS	Δ(*srl-recA*)306::Tn10(DE3), Tet^r/Cm^r)	Novagen
Plasmids		
pET21a	Vector containing a T7 promoter, Amp^r	Novagen
pBB04EL	Pir-dependent suicide vector containing *pagL*::TnphoA 5′ fusion junction	Ref. 51
pWKS30	Low copy vector	Ref. 70
pBluescript KS II+	*lacZ*, Amp^r	Stratagene
pPagL	pET21a containing *pagL*	This work
pWLP23	pWKS30 containing *pagL*	This work
pWLP21	pBluescript KS II+ containing *pagL*	This work
pWLP24	Δ*pagL* exchange vector	This work
PKAS32	Allelic exchange vector	Ref. 45

by the addition of fresh preequilibrated lower phase. The lower phases were pooled and dried under a stream of N_2. Finally, the dried lipid was resuspended in 50 mM Hepes, pH 7.5, and stored at −20 °C. To prepare Kdo_2-[4′-^{32}P]lipid IV_A, the purified *E. coli* Kdo transferase was added to the system immediately after the 4′-kinase, as previously described (36). The Kdo_2-[4′-^{32}P]lipid IV_A was isolated as described above with the exception that 50 mM ammonium acetate adjusted to pH 1.5 was used as the aqueous component instead of 0.1 M HCl in all Bligh/Dyer systems. The final yields of the desired radioactive lipid products ranged between 40 and 60 μCi from 100 μCi of [γ^{32}-P]ATP used as the starting material. Both lipid products were stored as aqueous dispersions at −80 °C and subjected to sonic irradiation for 1 min in a bath sonicator prior to use (36).

Preparation of Cell-free Extracts and Membranes—Typically, 100-ml cultures of bacteria were grown to an A_{600} of 1.0 at 37 °C and harvested by centrifugation at 7,000 × *g* for 15 min. All steps were carried out at 4 °C. Cell pellets were resuspended in 50 mM Hepes, pH 7.5, at a protein concentration of ~3–8 mg/ml and broken by passage through a French pressure cell at 18,000 p.s.i. The crude lysate was cleared by centrifugation at 7,000 × *g* for 15 min. Membranes were prepared by two centrifugation steps at 149,000 × *g* for 60 min with a wash of the crude membranes in 5 ml of 50 mM Hepes, pH 7.5, after the first centrifugation to ensure the removal of all cytosolic components. The final membrane pellet was resuspended in 50 mM Hepes, pH 7.5, at a protein concentration of ~5–10 mg/ml. Cytosol from the first 149,000 × *g* centrifugation was subjected to a second centrifugation step for complete removal of small membrane fragments. All samples were stored in aliquots at −80 °C, and protein concentrations were determined with bicinchoninic acid (38), with bovine serum albumin as the standard.

3-O-Deacylase Assay—The 3-*O*-deacylase activity was assayed under optimized conditions in a 10-μl reaction mixture containing 50 mM Hepes, pH 8.0, 0.1% Triton X-100, 0.5 M NaCl, and 10 μM [4′-^{32}P]lipid IV_A (50,000 cpm/nmol). Reaction tubes were incubated at 30 °C for the indicated times. The assays were stopped by spotting 5-μl portions of the reaction mixtures onto a silica gel 60 TLC plate. For further characterization of the 3-*O*-deacylase activity, the $PhoP^C$ *pagP*$^-$ *Salmonella* mutant strain (Table I) was used as the enzyme source to avoid other further acylation of the [4′-^{32}P]lipid IV_A substrate by PagP (31).

Thin Layer Chromatography—When [4′-^{32}P]lipid IV_A was employed as the substrate, the reaction products were separated using the solvent system chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v). For reactions containing Kdo_2-[4′-^{32}P]lipid IV_A as the substrate, plates were developed in chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v/v/v). Finally, reaction products from assays containing ^{32}P-labeled lipid X (39, 40) as the substrate were separated using the solvent chloroform/methanol/water/acetic acid (25:15:4:2, v/v/v/v). Reaction products were analyzed using a Molecular Dynamics PhosphorImager equipped with ImageQuant software. The enzyme activity was calculated by determining the percentage of the substrate converted to product, and the specific activity was expressed as nmol/min/mg.

Mild Alkaline Base Hydrolysis Using Triethylamine—The 3-*O*-deacylated lipid IV_A reaction product was generated in a 50-μl reaction mixture for 2 h, as described above, using membranes from the $PhoP^C$ *pagP*$^-$ *S. typhimurium* mutant strain. Mild base hydrolysis was carried out by the addition of triethylamine to a final concentration of 30%, and the reaction was incubated at 37 °C (41). At the indicated times, 3-μl portions of the reaction mixture were removed and mixed with 3 μl of water, after which 5 μl of the resulting mixture was spotted onto a silica gel 60 TLC plate and developed in chloroform/pyidine/88% formic acid/water (50:50:16:10, v/v/v/v). As a control, the [4′-^{32}P]lipid IV_A substrate was also subjected to triethylamine hydrolysis under the same conditions (41).

Separation of Inner and Outer Membranes—Membranes from various strains of *E. coli*, *S. typhimurium*, or *P. aeruginosa* were separated by isopycnic sucrose gradient centrifugation. First, washed membranes were prepared as described above and were resuspended in 10 mM

TABLE II
Oligonucleotides

Name	Sequence
T7PagLNde	5′-GCGCGCCATATGATGAAGAGAATATTT-3′
T7PagLBam	5′-GCGCGCGGATCCTCAGAAATTATAACT-3′
WLP22-*Eco*RI	5′-CTGAATTCGTAAGCGTGG-3′
WLP23-*Bam*HI	5′-CAGGGATCCTAAAACGTTC-3′
WLP33-*Kpn*I	5′-CCGGTACCACCTTTCATATTCA-3′
WLP34-*Eco*RI	5′-AAGAATTCTCTTCATATACACTCCACC-3′
WLP35-*Eco*RI	5′-AGGAATTCTTTCTGAAGTTGAATAAC-3′
WLP36-*Xba*I	5′-TTGTCTAGAGCCCTTTAGCATC-3′
T7 promotor	5′-TAATACGACTCACTATAGGG-3′
T7 terminator	5′-GCTAGTTATTGCTCAGCGG-3′

[a] Underlined bases represent restriction enzyme sites, as indicated in the primer designations.

Hepes, pH 7.0, containing 0.05 mM EDTA at a protein concentration of 5 mg/ml. Membranes were applied to a seven-step gradient, prepared as described by Guy-Caffey *et al*. (42, 43), and subjected to ultracentrifugation in a Beckman SW40.1 rotor for 19 h at 3 °C. The gradient was collected in ~0.5-ml fractions. Each fraction was then assayed for NADH oxidase as the inner membrane marker and for phospholipase A as the outer membrane marker, as previously described (44). The amount of protein in each fraction was determined using the bicinchoninic acid assay (38). Each fraction was also assayed for the 3-*O*-deacylase activity using the standard conditions described above.

Recombinant DNA Techniques—Plasmids were prepared using the Qiagen Spin Prep kit. DNA fragments were isolated from agarose gels using the Qiaex II gel extraction kit. T4 DNA ligase (Life Technologies, Inc.), restriction endonucleases (New England BioLabs), and shrimp alkaline phosphatase (U. S. Biochemical Corp.) were used according to the manufacturer's instructions.

Sequencing of pagL Gene—Initial sequence of the *pagL* region was obtained by sequencing pBB04EL, a plasmid containing the *pagL*::TnphoA 5′ fusion junction (30). Sequence was obtained in both directions using standard techniques with a Perkin-Elmer ABI Prism 377 automated DNA sequencer equipped with Sequencher 3.0 software. The *pagL* sequence was subsequently verified from chromosomal DNA cloned into pBluescript (pWLP21) as described below.

Construction of a PhoP^C pagL Deletion Mutant—A nonpolar deletion of greater than 95% of the coding sequence of *pagL* was created using PCR amplification of flanking DNA with *Pfu* (Stratagene) according to the manufacturer's instructions. The flanking DNA was subsequently cloned into the allelic exchange vector, pKAS32 (45), resulting in the plasmid pWLP24 containing the Δ*pagL* construct. Allelic exchange was performed in strain CS401, as has been described (46). Resolution of the integrant resulted in a Δ*pagL* strain CS586, which was verified using PCR and Southern blot analysis.

To create a nonpolar Δ*pagL* in a background that constitutively expresses PhoP/PhoQ, P22HT*int* bacteriophage was grown on the CS401 pWLP24 integrant, and the integrated plasmid was transduced into the PhoP^C streptomycin-resistant strain CS491. Resolution of the integrated plasmid resulted in a PhoP^C Δ*pagL* strain, CS584. The successful deletion of *pagL* was verified by PCR and Southern blot analysis.

Construction of pWLP21 and pWLP23—The *pagL* gene and its flanking sequences, including 79 base pairs upstream and 168 base pairs downstream, were amplified by PCR from *S. typhimurium* 14028 genomic DNA with *Pfu* Turbo (Stratagene) according to the manufacturer's instructions. The PCR product was cloned into both the high copy vector pBluescript KS II$^+$ (pWLP21) and the low copy vector pWKS30 (pWLP23) (Table II).

Overexpression of PagL behind a T7 Promoter—The *pagL* gene was amplified by PCR using *Pfu* Turbo (Stratagene) according to the manufacturer's instructions, using *S. typhimurium* 14028 genomic DNA as the template. The PCR product was cloned into pET21a(+) under the control of the T7 promoter to overexpress the enzyme giving the construct, pPagL. The *pagL* construct was transformed into BLR(DE3)/pLysS (Novagen) for overexpression of PagL. First, a single colony of *E. coli* BLR(DE3)/pLysS containing pPagL was inoculated into 20 ml of LB medium and grown to an A_{600} = 0.8. The culture was then used to inoculate 1 liter of fresh LB medium, and at A_{600} of ~0.6, the cells were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. Crude extracts, membrane-free cytosol, and washed membranes were prepared as described above.

Protein Gel Electrophoresis—Protein extracts were analyzed using the Bio-Rad Protean II XI apparatus. Samples containing 40 μg of protein were solubilized in ⅓ volume of 2× SDS buffer and heated for 5 min. Samples were applied to a 1.5-mm-thick 15% polyacrylamide SDS gel with a 4% stacking gel (22.2 × 20 cm) and were subjected to electrophoresis at 100 V. Gels were stained with Coomassie Blue dye (2.5 mg/ml) in water/methanol/acetic acid (6:4:1, v/v/v) and were then destained with water/methanol/acetic acid (6:4:1, v/v/v). Prestained low range standards from Bio-Rad were used to estimate molecular weight.

Protein Microsequencing—Protein from the outer membrane fraction of the *E. coli* expression strain BLR(DE3)pLysS containing the T7 *pagL* construct (pPagL) was separated by SDS-polyacrylamide gel electrophoresis as described above. The portion of the gel containing the PagL protein was excised, and the protein was transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) in 10 mM CAPS, pH 11, in 10% methanol at 15 V for 30 min using the Bio-Rad Semi-Dry Transfer apparatus. Transferred protein was stained with 0.1% Ponceau S in 1% acetic acid for 1 min and then destained with 1% acetic acid for 10 min. The PagL protein band was excised, rinsed three times with distilled water, and subjected to high sensitivity protein microsequencing on ABI model 492A Procise Sequencer at the University of Massachusetts Medical School Core Laboratory for Protein Microsequencing and Mass Spectrometry (Worcester, MA).

Large Scale Isolation of Lipid A from E. coli Cells Expressing the Heterologous pagL Gene—Cultures (100 ml) of the *E. coli* strain XL-1 Blue were grown in LB medium at 37 °C containing either pBluescript (Stratagene) or pWLP21. After A_{600} of ~1.0 was reached, cells were harvested, resuspended in 80 ml of phosphate-buffered saline (47), and frozen prior to lipid A isolation. Lipid A was released from cells and purified as previously described and stored frozen at −80 °C (28, 48).

Mass Spectrometry of Lipid A Species—Spectra were obtained in the negative linear mode using a matrix-assisted laser desorption/ionization time of flight Bruker BiflexIII mass spectrometer (Bruker Daltonics, Inc., Billerica, MA). Each spectrum was the average of 100 shots. Lipid samples were dissolved in chloroform/methanol (4:1, v/v) before mixing with the matrix (lipid/matrix; 9:1, v/v). The matrix consisted of saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% ammonium sulfate. The mixtures were allowed to dry at room temperature on the sample plate prior to mass analysis.

RESULTS

A PhoP/PhoQ-dependent Deacylase in S. typhimurium Membranes—As shown in Fig. 3*A*, membranes from a strain of *S. typhimurium* in which the PhoP transcriptional regulatory protein is constitutively active (PhoP^C) (49) are capable of converting the lipid A precursor, [4′-^{32}P]lipid IV$_A$, to a more hydrophilic product (*lane 3*) with an R_F of 0.28. This product, denoted as deacyl-IV$_A$, is the same as that produced by the 3-*O*-deacylases of *Rhizobium etli* and of *P. aeruginosa*, previously characterized by Basu *et al.* (41). The faster migrating species, lipid IV$_B$, arises by the addition of a palmitate chain to the amide-linked β-hydroxymyristate residue at position 2 of lipid IV$_A$ (31, 32), catalyzed by the *pagP* gene product (31) (Fig. 2). The additional radioactive lipid shown in *lane 3* (designated deacyl-IV$_B$) results from both the addition of a palmitate chain and 3-*O*-deacylation of [4′-^{32}P]lipid IV$_A$ (Fig. 2). When membranes from a PhoP^C strain, also harboring a *pagP* mutation (31), were used, both products containing palmitate were eliminated (*lane 5*).

Membranes from wild-type *S. typhimurium* grown in LB broth (Fig. 3, *lane 2*) or in minimal medium (pH 7.4) containing 10 mM Mg^{2+} (Fig. 3, *lane 8*), conditions under which the PhoP/PhoQ system is inactive, showed little or no deacylase activity. In membranes from cells grown in minimal medium (pH 7.4) containing 10 μM Mg^{2+}, however, the 3-*O*-deacylase was very active (Fig. 3, *lane 9*), consistent with PhoP/PhoQ regulation (5, 12). The specific activity of the deacylase in membranes of either the PhoP^C strain or of wild-type *S. typhimurium* cells grown in limiting Mg^{2+} was ~0.50 nmol/min/mg. Membranes from a PhoP^C strain with a *pagP* deletion displayed even higher specific activity (~1.35 nmol/min/mg). Since palmitoylation of lipid IV$_A$ by PagP represents a competing reaction in *Salmonella* membranes, a PhoP^C *pagP*$^-$ strain was routinely used for further characterization of the deacylase.

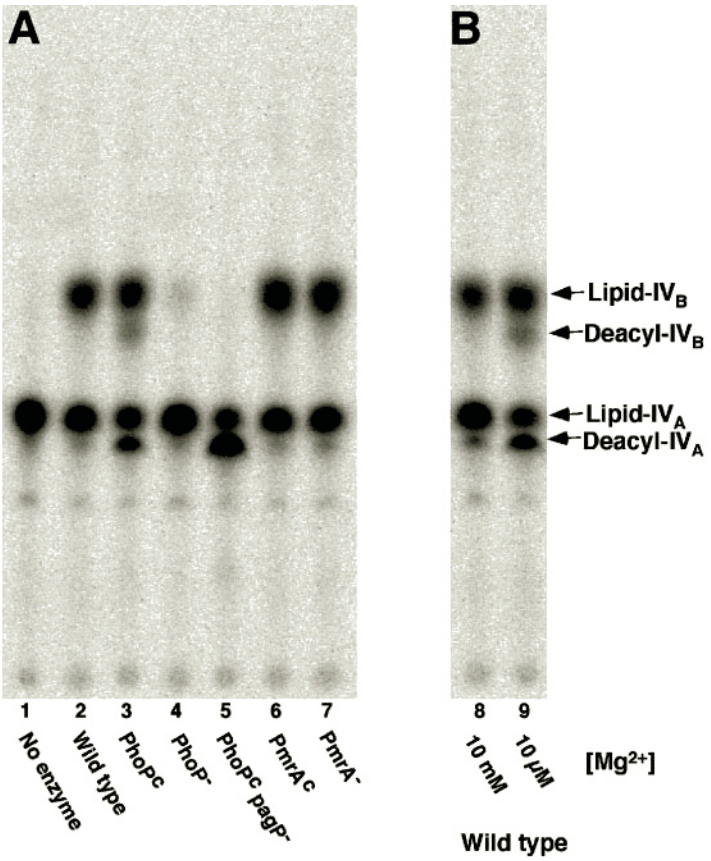

FIG. 3. **Identification of a 3-*O*-deacylase in *S. typhimurium* membranes and its regulation.** *A*, membranes from the indicated strains of *S. typhimurium* (Table I) were assayed for 3-*O*-deacylase activity using assay conditions described under "Experimental Procedures." The protein concentration was 0.1 mg/ml, and assays were carried out for 30 min at 30 °C. Each reaction contained 10 μM [4′-^{32}P]lipid IV$_A$, and the products were separated by TLC and detected with a PhosphorImager. *B*, wild-type *S. typhimurium* membranes from cells grown at pH 7.4 in either 10 μM or 10 mM Mg^{2+} were assayed for deacylase activity under the conditions described above. R_F values for the various metabolites were determined and are listed in ascending order: R_{F1} = 0.28, R_{F2} = 0.32, R_{F3} = 0.42, RR_{F4} = 0.49.

The PmrA/PmrB two-component system was previously implicated in the covalent modification of lipid A with phosphoethanolamine and 4-aminoarabinose (26, 27, 30) and was therefore tested as a regulator of the 3-*O*-deacylase. Membranes from an *S. typhimurium* strain in which the transcriptional regulatory protein PmrA is constitutively active (PmrA^C) showed no deacylase activity (Fig. 3, *lane 6*). Also, growth in minimal medium at a pH of 5.8, a condition known to activate the PmrA/PmrB system (5), failed to induce deacylase activity unless the Mg^{2+} concentration was also limiting (10 μM) (data not shown). As previously demonstrated by Basu *et al.* (41), *E. coli* membranes do not possess a deacylase when cells are grown in broth. Similarly, we found that growth in minimal medium containing 10 μM Mg^{2+} at pH 7.4 or 5.8 was unable to induce deacylase activity in membranes of *E. coli* MC1061 or W3110 (data not shown).

Mild Alkaline Hydrolysis of the S. typhimurium 3-O-Deacylase Reaction Product—Hydrolysis of lipid A and its precursors with the mild base triethylamine at 30 °C results in the selective removal of the 3-*O*-linked β-hydroxymyristoyl group, followed by gradual removal of the 3′-*O*-linked fatty acyl moiety (41). The triethylamine deacylation products of [4′-^{32}P]lipid IV$_A$ are easily separated by TLC, are well characterized, and can be used as standards (41). The [4′-^{32}P]lipid IV$_A$ substrate (Fig. 4*A*) and the 4′-^{32}P hydrophilic reaction product generated by PhoP^C *pagP*$^-$ *Salmonella* membranes (Fig. 4*B*) were treated in parallel with triethylamine. As shown in Fig. 4*A*, treatment of [4′-^{32}P]lipid IV$_A$ with triethylamine results in gradual removal of both ester-linked fatty acids, giving rise to the 3- or 3′-*O*-deacylated materials as intermediates and the doubly *O*-deacylated species as the final product. The hydrophilic species generated from [4′-^{32}P]lipid IV$_A$ by PhoP^C *pagP*$^-$ *Salmonella* membranes (Fig. 4*B*) migrates the same as the 3-*O*-deacylated [4′-^{32}P]lipid IV$_A$ standard (Fig. 4*A*). Further treatment of this product (Fig. 4*B*) with triethylamine results in formation of a compound migrating like doubly *O*-deacylated [4′-^{32}P]lipid IV$_A$. These results demonstrate that the hydrophilic material generated from [4′-^{32}P]lipid IV$_A$ by PhoP^C *pagP*$^-$ *Salmonella* membranes is probably a 3-*O*-deacylated species and does not contain some other hydrophilic unit that slows its migration during TLC analysis.

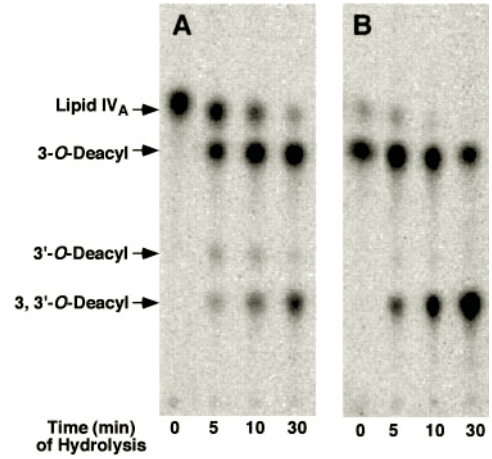

FIG. 4. **Triethylamine hydrolysis of [4′-^{32}P]lipid IV$_A$ and the hydrophilic product generated by membranes of strain CS330.** As described under "Experimental Procedures," the [4′-^{32}P]lipid IV$_A$ substrate (10 μM) was incubated either with or without membranes from the PhoP^C *pagP*$^-$ strain (CS330) under standard assay conditions using 1 mg/ml membranes for 2 h at 30 °C. Following the reaction, the [4′-^{32}P]lipid IV$_A$ and the hydrophilic product generated by CS330 membranes were treated with triethylamine for the indicated times, after which a portion of each sample was separated by TLC and subjected to PhosphorImager analysis.

Assay Conditions and Catalytic Properties of the PhoP/PhoQ-regulated 3-O-Deacylase—The 3-*O*-deacylation of [4′-^{32}P]lipid IV$_A$ is absolutely dependent upon the presence of the

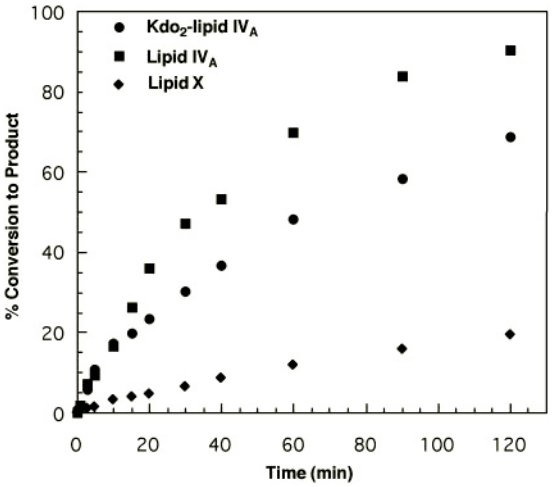

FIG. 5. **Substrate specificity and time dependence of the 3-*O*-deacylase.** Deacylase activity was assayed under standard assay conditions with either 10 μM [4′-^{32}P]lipid IV_A, Kdo_2-[4′-^{32}P]lipid IV_A, or ^{32}P-labeled lipid X. Membranes at 1.0 mg/ml from CS330 ($PhoP^C$, *pagP*$^-$ *S. typhimurium*) served as the enzyme source. Reaction products were separated by TLC as described under "Experimental Procedures" and subjected to PhosphorImager analysis.

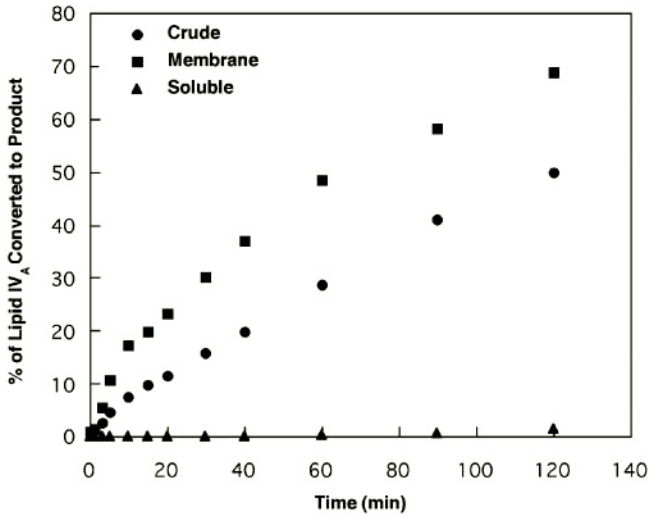

FIG. 6. **3-*O*-Deacylase activity is associated with the membrane fraction *of S. typhimurium*.** The 3-*O*-deacylase activity of the $PhoP^C$ *pagP*$^-$ strain CS330 was assayed under standard assay conditions with 10 μM [4′-^{32}P]lipid IV_A. Crude extract, cytosol, or membranes served as the enzyme source. The reaction products from the indicated times were separated by TLC and subjected to PhosphorImager analysis.

nonionic detergent, Triton X-100, with optimal activity at 0.1%. The pH optimum is 8.0, but significant activity is observed from pH 5.5 to 9.0. Divalent cations are not required. EDTA and EGTA have no effect. Increased activity is observed with higher ionic strength. Accordingly, 0.5 M NaCl is included in the assay system (data not shown). The substrate specificity of the enzyme is relatively broad (Fig. 5). The deacylase does not require the Kdo moiety for activity, showing a slightly higher activity with 10 μM [4′-^{32}P]lipid IV_A than with 10 μM Kdo_2-[4′-^{32}P]lipid IV_A. The relative rate of deacylation is decreased ~10-fold when the monosaccharide precursor, lipid X (40, 50), is used as the substrate (Fig. 5) at 10 μM. Product formation by the deacylase with 10 μM [4′-^{32}P]lipid IV_A is linearly dependent upon both protein concentration (data not shown) and times less than 10 min (Fig. 5). Interestingly, we have so far been unable to demonstrate activity with hexa-acylated lipid A as the substrate (data not shown), raising an interesting paradox in light of the subcellular localization of the enzyme (see below).

The assay conditions for the *Rhizobium leguminosarum*/*etli* 3-*O*-deacylase (41) differ from those for the *S. typhimurium* enzyme with regard to pH optimum, Triton X-100 dependence, and requirement for divalent cations. Optimal deacylation conditions for *P. aeruginosa* membranes resemble those for the *Salmonella* enzyme (data not shown). However, the 3-*O*-deacylase of wild-type *P. aeruginosa* PAO1 is present in membranes prepared from cultures grown in LB broth (41). The deacylase activity of PAO1 is not increased in membranes prepared from cells grown in the presence of low Mg^{2+} (10 μM) (data not shown). Last, PhoP null mutants of *P. aeruginosa* PAO1 still make 3-*O*-deacylated lipid A species when grown in the presence of low Mg^{2+}, whereas modifications with 4-aminoarabinose and palmitate are lost (11). The combined data suggest that the 3-*O*-deacylase of PAO1 is regulated differently than the *Salmonella* enzyme.

Outer Membrane Localization of the S. typhimurium 3-O-Deacylase—The deacylase activity of *S. typhimurium* is localized in the particulate fraction (Fig. 6). Further separation by isopycnic density gradient centrifugation reveals that the enzyme is mainly an outer membrane protein (Fig. 7), although a small but significant fraction of the activity is also seen in the inner membrane. NADH oxidase serves as the inner membrane marker, whereas phospholipase A activity is used to locate outer membrane fragments (Fig. 7). Besides PagP (31), the 3-*O*-deacylase of *S. typhimurium* is only the second example of

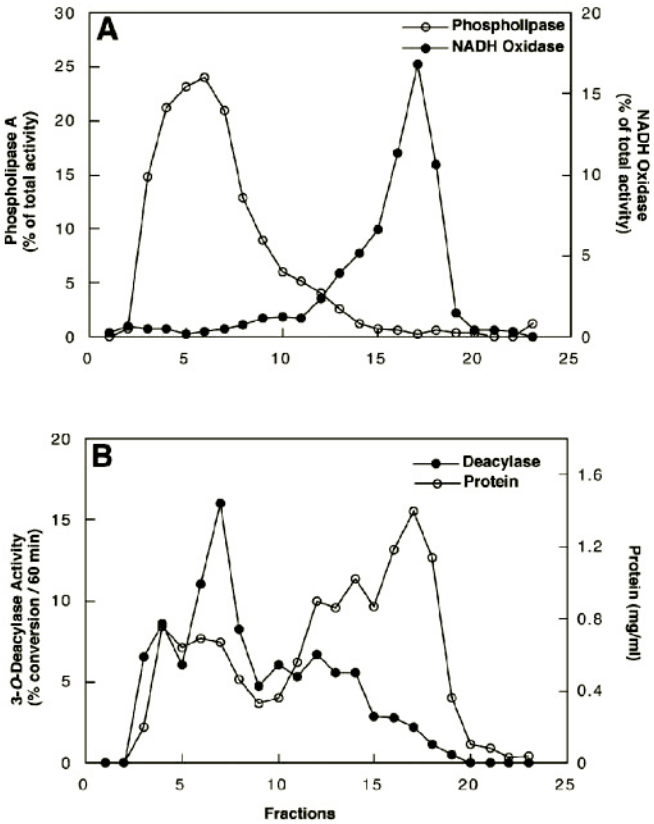

FIG. 7. **Outer membrane localization of the PhoP/PhoQ regulated 3-*O*-deacylase of *S. typhimurium*.** Membranes isolated from the $PhoP^C$ *pagP*$^-$ strain (CS330) were separated by isopycnic sucrose density gradient centrifugation, and ~0.5-ml fractions were collected. *A*, phospholipase A and NADH oxidase activities were assayed as markers for outer and inner membrane fragments, respectively, and expressed as a percentage of the total activity across the gradient. *B*, protein concentration and 3-*O*-deacylase activity were assayed for each fraction.

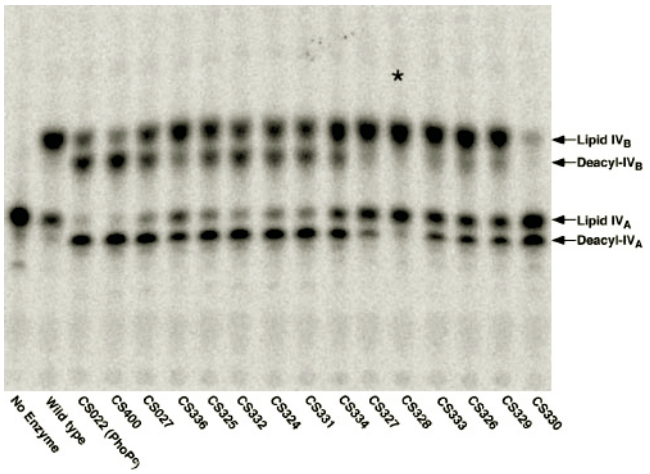

FIG. 8. **Screening of extracts of *S. typhimurium* mutants defective in PhoP-activated genes for loss of 3-*O*-deacylase activity.** Membranes from various PhoP^C strains of *S. typhimurium*, each containing a separate TnphoA transposon insertion in a different *pag* gene, were assayed for 3-*O*-deacylase activity. The source of the individual *pag* knockouts is indicated in Table I. Membranes from wild-type *S. typhimurium* and a PhoP^C *S. typhimurium* strain (CS022) served as the negative and positive controls, respectively. Assays were performed under the standard conditions for 1 h at 30 °C using 1 mg/ml membranes and 10 μM [4′-^{32}P]lipid IV$_A$. Products were separated by TLC and visualized by PhosphorImager analysis.

```
         10          20 ⇓        30          40
MKRIFIYLLL PCAFACSAND NVFFGKGNKH QISFAAGESI
CCCCEEEECC CCCCCCCCCC CEEECCCCCC EEEEECCHHH

         50          60          70          80
RRGGVEHLYT AFLTYSEPSD FFFLQARNNL ELGGFKAKGS
HHCCCCEEEE EEECCCCCCC EEEECCCCCC CCCCEEECCC

         90         100         110         120
DDCSKHSGSV PCNKYNQGVL GISKDVALVH FAGIYTGIGL
CCCCCCCCCC CCCCCCCCEE ECCCCEEEEE EECCCCCCCC

        130         140         150         160
GAYIKSKSRD DMRVNSAFTF GEKAFLGWNF GAFSTEAYIR
EEEEECCCCC CCCCEEECCC CCCCEEECCC CCCCCEEEEE

        170         180
HFSNGSLTDK NSGHNFVGAS ISYNF
ECCCCCCCCC CCCCCEEEEE ECCCC

H = Helix
C = Coil
E = Beta Sheet
```

FIG. 9. **Sequence of the *S. typhimurium* PagL protein and its secondary structure prediction.** The 3-*O*-deacylase protein sequence was deduced from the *pagL* gene sequence. The cleavage site of the *N*-terminal signal peptide, designated by a *downward arrow*, occurs between amino acids 20 and 21 (AND-NVF) as evidenced by N-terminal amino acid sequencing.

an outer membrane protein involved in lipid A modification. The 3-*O*-deacylase of *P. aeruginosa* is also localized mainly in the outer membrane fraction (data not shown).

Identification of the Structural Gene (pagL) Encoding the 3-O-Deacylase of S. typhimurium—Previously, several *S. typhimurium* PhoP-activated (*pag*) genes were mutated using a TnphoA transposon in a PhoP^C background (51). Since the deacylase is greatly induced in the PhoP^C background, loss of enzymatic activity in the membranes of a particular *pag* mutant could reveal the structural gene encoding the deacylase or, alternatively, additional regulatory protein(s) necessary for deacylase expression. Membranes of strains containing single, distinct TnphoA mutations (Table I) were prepared and assayed for 3-*O*-deacylase activity. Out of 14 available *pag* mutants, only one (CS328) showed a loss of deacylase activity (Fig. 8). CS328 contains an insertion in a previously unreported PhoP activated gene, designated *pagL*. The palmitoyltransferase (PagP) activity is still present in CS328 and all other *pag* mutant strains except for CS330, which harbors a *pagP* insertion.

Using the DNA sequence of the transposon as the starting point, the sequence of the inactivated *pag* gene (*pagL*) was determined. A novel open reading frame was identified (Fig. 9). Comparison of the predicted PagL amino acid sequence with putative proteins in the nonredundant and incomplete microbial data bases using the BLASTp or tBLASTn programs (52, 53) revealed no obvious homologues of PagL except in other strains of *Salmonella*. Analysis of the amino acid sequence indicated the presence of a 15-amino acid type I signal peptide using the Signal-P Program (54), supporting the view that the protein may be localized to the outer membrane fraction. The uncleaved protein has a predicted molecular mass of ~20 kDa and a pI of ~9.0.

A nonpolar deletion compromising greater than 95% of the *pagL* coding sequence was generated in the *S. typhimurium* strain CS401 (46), followed by transduction of the mutation into the PhoP^C streptomycin-resistant strain, CS491. Membranes of CS584 (*phoP*^{C}Δ*pagL*) contained no deacylase, as shown in Fig. 10, *lane 2*, when assayed under optimal conditions at 0.01 mg/ml for 10 min or even at 2 mg/ml (data not

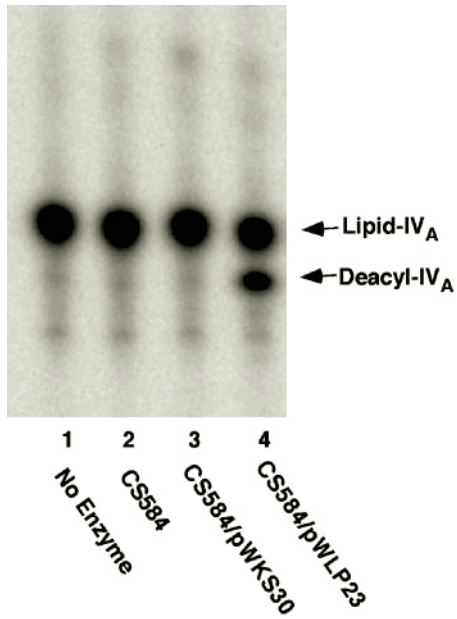

FIG. 10. **Reconstitution of 3-*O*-deacylase activity in membranes of a PhoP^{C}Δ*pagL* mutant by introduction of a *pagL*-covering plasmid.** Membranes from the PhoP^C Δ*pagL* mutant (CS584), CS584 containing the vector control (pWSK30), or CS584 containing the plasmid with the *pagL* gene (pWLP23) were assayed for deacylase activity. Assays were performed under standard conditions using 0.01 mg/ml membrane protein for 10 min.

shown). To demonstrate that recovery of deacylase activity was dependent upon the *pagL* gene, *pagL* and its flanking 5′ and 3′ sequences were cloned into the low copy vector, pWKS30, and the resulting plasmid was named pWLP23. The 3-*O*-deacylase activity of CS584 was recovered upon transformation with pWLP23 (Fig. 10, *lane 4*). Introduction of the vector control had no effect on deacylase activity (Fig. 10, *lane 3*). Although these data are strongly suggestive, they do not unequivocally prove that *pagL* is the structural gene encoding the deacylase.

Heterologous Expression of pagL in E. coli—To obtain additional evidence that *pagL* is the structural gene for the 3-*O*-

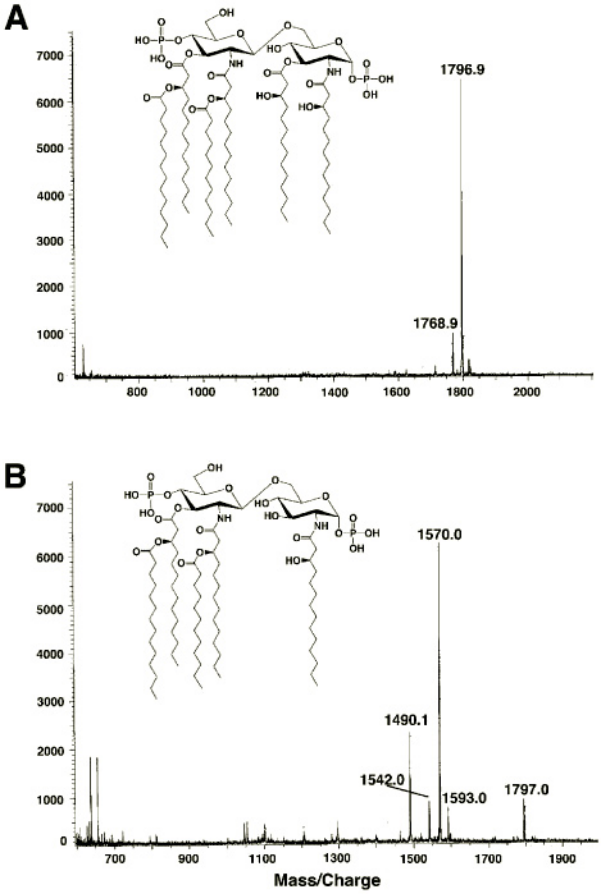

FIG. 11. **Matrix-assisted laser desorption/ionization time of flight mass spectrometry of lipid A from *E. coli* expressing a heterologous *pagL* gene.** The lipid A of the *E. coli* XL-1 Blue strain containing either pBluescript (*A*) or pWLP21 (pBluescript with *pagL*) (*B*) was isolated using published protocols (48). The lipid A samples were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry in the negative linear ion mode.

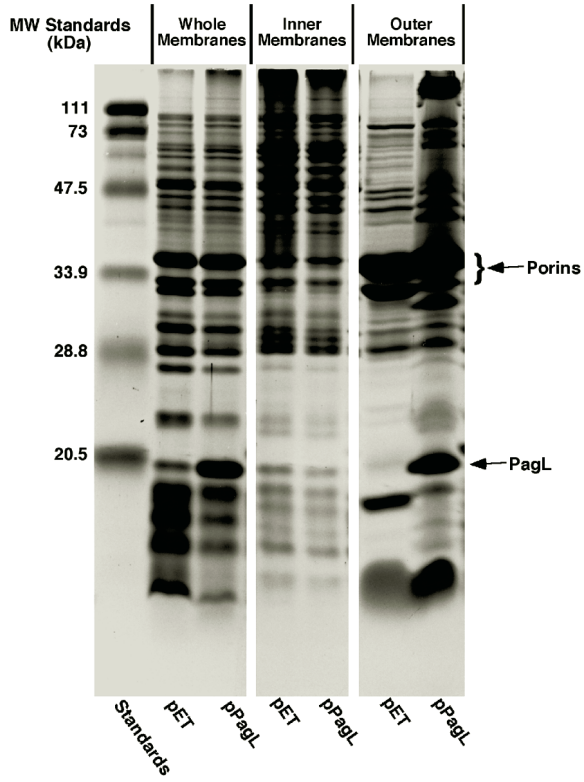

FIG. 12. **Overexpression and outer membrane localization of PagL in the *E. coli* BLR(DE3)pLysS background.** Membranes from the BLR(DE3)/pLysS expression strain, containing either pET21a or pPagL plasmids, were isolated, and 40-μg samples of protein were analyzed by SDS-polyacrylamide gel electrophoresis. The inner and outer membranes of the strains were separated by isopycnic sucrose density gradient centrifugation, and 0.5-ml fractions were collected as described under "Experimental Procedures." For the fractions shown, 40-μg samples of protein were analyzed, and the gel was stained with Coomassie Blue. The positions of the molecular weight standards, PagL, and the porins are indicated.

deacylase, a heterologous *E. coli* expression system was established. Both the control pBluescript vector and pWLP21 were transformed into *E. coli* strain XL-1 Blue, and the lipid A of each strain was isolated and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry. The lipid A of XL-1 Blue, containing the control vector, consisted primarily of the hexa-acylated *bis*-phosphate species that is typically seen in *E. coli* K12 strains (28, 48), with $[M - H]^-$ at m/z 1796.9 atomic mass units in the negative mode (Fig. 11*A*). However, upon overexpression of *pagL*, the $[M - H]^-$ of the predominant lipid A species was detected at m/z 1570 atomic mass units in the negative mode, corresponding to the loss of one β-hydroxymyristate residue from the major species seen in the vector control (Fig. 11*B*). The additional species at m/z 1490 atomic mass units corresponds to loss of the phosphate group from the 1-position of the 3-*O*-deacylated lipid A, most likely a fragment ion, since analysis of the sample by TLC followed by charring showed no such lipid. Based upon mass spectroscopy, it can be concluded that selective lipid A deacylation occurs in living cells of this heterologous construct at the 3-position. Loss of β-hydroxymyristate at the 3′-position would also lead to the loss of the secondary myristate chain, yielding a tetra-acylated lipid A variant with a molecular weight of 1361.7, a species not seen in Fig. 11. Furthermore, heterologous expression of *pagL* in *E. coli* did not slow down cell growth (data not shown).

There are no homologues of the *pagL* gene in *E. coli*, consistent with the observation that there is no 3-*O*-deacylase activity in *E. coli* membranes, irrespective of growth or assay conditions. The above data further support the view that *pagL* of *S. typhimurium* is the structural gene for the 3-*O*-deacylase.

T7 Promoter-driven Overexpression of PagL and Its Localization in the Outer Membrane—Overproduction of PagL protein was achieved by cloning the PCR-amplified *pagL* gene behind a T7 promoter into the expression vector pET21a, giving the plasmid pPagL. Membranes isolated from the *E. coli* expression strain BLR(DE3)pLysS containing either pET21a or pPagL were assayed for 3-*O*-deacylase activity. The specific activity of the deacylase in membranes of PhoP^C *S. typhimurium* was 0.50 nmol/min/mg *versus* 155 nmol/min/mg in the induced T7 overexpression system, a 300-fold increase in activity. Membranes from the BLR(DE3)pLysS vector control strain were inactive. As in PhoP^C *S. typhimurium* (Fig. 7), the overexpressed PagL in *E. coli* was located largely in the outer membrane (Fig. 12). Comparison of the outer membranes of BLR(DE3)pLysS containing either the control vector or pPagL shows overproduction of a protein migrating with the predicted molecular mass of mature PagL (18 kDa) (Fig. 12) in the latter. To verify the presence of the signal peptide predicted by the Signal-P program and to determine the cleavage site, PagL protein from outer membranes of BLR(DE3)pLysS/pPagL was electroblotted to a polyvinylidene difluoride membrane and subjected to microsequencing. The sequence of the first 10 amino acid residues was NVFFGKGNKH, indicating that cleavage of the signal peptide occurs between amino acid resi-

dues 20 and 21 (AND-NVF) of PagL rather than between amino acids 15 and 16 as predicted by Signal-P (54) (Fig. 9).

Thermal Stability of PagL—The outer membrane enzyme PagP resists thermal denaturation (31), since it retains ~65% of its enzymatic activity after a 10-min preincubation at 100 °C (32). PagL displays similar behavior (data not shown). The unusual thermal stability of both enzymes may be due to their relatively low molecular weights.

DISCUSSION

Over the past 15 years, nine constitutive enzymes of lipid A biosynthesis have been identified in *E. coli* (13, 14, 55). With few exceptions, single copies of the corresponding structural genes are present in all Gram-negative bacteria. However, the enzymes and genes responsible for the covalent modifications of lipid A (Fig. 1), which are associated with bacterial virulence and polymyxin resistance, are not yet fully characterized (4, 26, 27, 56, 57). It has been demonstrated that these modifications are controlled by the PhoP/PhoQ and PmrA/PmrB two-component regulatory systems (4, 26, 27, 56, 57) and also can be induced with metavanadate in *E. coli* (28). The enzymatic function of the PhoP-activated lipid A palmitoyltransferase, PagP, was recently established in our laboratories (31). Furthermore, the biosynthesis of the PhoP/PhoQ-dependent *S*-2-hydroxymyristate moiety found in the lipids A of *S. typhimurium* and certain other pathogenic bacteria was shown to depend upon a novel lipid A hydroxylase homologue, designated LpxO (58).

We now present the initial characterization, cloning, and overexpression of another lipid A-modifying enzyme, PagL, an unusual PhoP/PhoQ-activated lipase that selectively removes the ester-linked 3-*O*-hydroxyacyl chains of certain lipid A precursors. The *S. typhimurium* lipid A 3-*O*-deacylase activity is under the control of the PhoP/PhoQ two-component regulatory system and was discovered using an *in vitro* assay with the tetra-acylated lipid A precursor, [4′-^{32}P]lipid IV_A, as the substrate (Fig. 3). By assaying extracts of individual $PhoP^C$ *S. typhimurium* strains harboring insertion mutations in 14 separate *pag* loci, the gene coding for the 3-*O*-deacylase (*pagL*) was found (Fig. 8). Complementation of a $PhoP^C$ Δ*pagL S. typhimurium* mutant with a low copy *pagL* plasmid restored deacylase activity. Heterologous expression of *pagL* in *E. coli*, an organism with no deacylase activity of its own and no *pagL* homologue in its genome, resulted in the appearance of robust deacylase activity in extracts and in loss of the *R*-3-hydroxymyristate moiety at position 3 in 90% of the lipid A molecules. There was no associated impairment of cell growth. These data, taken together with the sequencing of the overproduced PagL protein, demonstrate that *pagL* is the structural gene for the 3-*O*-deacylase.

Like the palmitoyltransferase PagP (31), the 3-*O*-deacylase PagL is a small, thermally stable enzyme, and it is associated with outer membrane enzyme as judged by the following observations. 1) Deacylase catalytic activity was detected mostly in outer membrane fragments of a $PhoP^C$ strain of *S. typhimurium* by assay with the substrate lipid IV_A. 2) Separation of membranes from an *E. coli* strain overexpressing *pagL* behind a T7 promoter showed that the recombinant protein was largely recovered in the outer membrane fractions, as judged by SDS-polyacrylamide gel electrophoresis (Fig. 12). 3) The overexpressed PagL protein was missing its type I signal peptide, as shown by *N*-terminal sequencing of the outer membrane-associated band. Because of their small sizes, both PagP (~19 kDa) and PagL (~18 kDa) may adopt the smallest possible β-barrel conformation that is characteristic of outer membrane proteins, consisting of only eight anti-parallel β-strands (59). Analysis of the PagL amino acid sequence (Fig. 9) with programs for predicting secondary structure reveals significant β-sheet domains. PagL is only one of four outer membrane enzymes characterized to date (31, 60).

No homologues of the *S. typhimurium* 3-*O*-deacylase were found in the nonredundant or unfinished microbial data bases, except in *S. typhi* and *S. paratyphi*, although other Gram-negative bacteria are known to contain 3-*O*-deacylated lipid A species (61, 62). A related lipid A lipase activity was previously demonstrated to be present in membranes of the nitrogen-fixing bacteria *R. leguminosarum* and *R. etli* (41), which are known to contain 3-*O*-deacylated lipid A species (63, 64). However, the gene encoding the 3-*O*-deacylase activity of *R. etli* is unknown, and the *R. etli*/*leguminosarum* enzyme requires divalent cations for activity (41), whereas PagL does not. Other Gram-negative bacteria that contain partially 3-*O*-deacylated lipid A include the pathogen *P. aeruginosa* (11, 61), which also possesses lipid A 3-*O*-deacylase activity (41) localized within its outer membrane.[2] Structural characterization of the lipid A from *Helicobacter pylori* by mass spectroscopy (65) indicates partial deacylation of the 3′-*O*-linked fatty acyl chain. Structural studies of lipid A from *Porphyromonas gingivalis* revealed that both the 3- and 3′-ester-linked fatty acids are partially removed (66). One would therefore expect additional lipid A lipases to be present in these organisms, but no obvious homologues of PagL were detected by BLASTp or PSI-BLAST searches (53) in any of these bacteria, suggesting the existence of additional, structurally distinct 3-*O*-deacylases.

The key remaining questions about PagL concern its biological function in *Salmonella* and the significance of its regulation during pathogenesis. A systematic study comparing the lipids A of diverse *Salmonella* strains by mass spectroscopy demonstrated partial absence of the *R*-3-hydroxymyristate substituent in many cases (62). Furthermore, small amounts of 3-*O*-deacylated lipid A species are detected among the lipid A precursors that accumulate in Kdo-deficient, temperature-sensitive mutants of *S. typhimurium*.[2] However, PhoP/PhoQ regulation of lipid A deacylation in *Salmonella* was not observed in previous studies comparing the lipid A structures of wild-type and *phoP* mutants (12), possibly because optimal conditions for inducing the deacylase in cells were not used. Unless a low copy *pagL*-bearing plasmid is introduced into *S. typhimurium* one does not see significant 3-*O*-deacylation of lipid A in cells under standard PhoP/PhoQ-activating growth conditions.[3] Perhaps additional as yet unknown signals are required for proper functioning of chromosomally encoded PagL.

We propose that the 3-*O*-deacylation of lipid A by a bacterium that is in the process of infecting an animal might result in a lower or altered immunological response, possibly aiding the bacterium in establishing a prolonged infection. It is well known that the presence of the phosphate groups at positions 1 and 4′ and the number and type of fatty acyl chains play a critical role in determining the immunological activity of lipid A (67, 68). As noted above, the lipid As of *H. pylori* and *P. gingivalis* are partially deacylated, and the lipids A of both organisms display significantly lower biological activities relative to other lipid A species (65, 66). The characterization of PagL mutants with respect to their pathogenesis and the analysis of various endotoxin-related activities of 3-*O*-deacylated lipid A species should help to clarify the functions of PagL.

Acknowledgments—M. S. T. thanks K. J. Babinski, S. S. Basu, and M. I. Kanipes for advice and help during the completion of this work.

[2] S. Trent and C. R. H. Raetz, unpublished results.

[3] M. S. Trent, W. Pabich, S. I. Miller, and C. R. H. Raetz, unpublished observations.

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