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Lipid Trafficking Controls Endotoxin Acylation in Outer Membranes of *Escherichia coli**

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The biogenesis of biological membranes hinges on the coordinated trafficking of membrane lipids between distinct cellular compartments. The bacterial outer membrane enzyme PagP confers resistance to host immune defenses by transferring a palmitate chain from a phospholipid to the lipid A (endotoxin) component of lipopolysaccharide. PagP is an eight-stranded antiparallel β-barrel, preceded by an N-terminal amphipathic α-helix. The active site is localized inside the β-barrel and is aligned with the lipopolysaccharide-containing outer leaflet, but the phospholipid substrates are normally restricted to the inner leaflet of the asymmetric outer membrane. We examined the possibility that PagP activity *in vivo* depends on the aberrant migration of phospholipids into the outer leaflet. We find that brief addition to *Escherichia coli* cultures of millimolar EDTA, which is reported to replace a fraction of lipopolysaccharide with phospholipids, rapidly induces palmitoylation of lipid A. Although expression of the *E. coli pagP* gene is induced during Mg^{2+} limitation by the *phoPQ* two-component signal transduction pathway, EDTA-induced lipid A palmitoylation occurs more rapidly than *pagP* induction and is independent of *de novo* protein synthesis. EDTA-induced lipid A palmitoylation requires functional MsbA, an essential ATP-binding cassette transporter needed for lipid transport to the outer membrane. A potential role for the PagP α-helix in phospholipid translocation to the outer leaflet was excluded by showing that α-helix deletions are active *in vivo*. Neither EDTA nor Mg^{2+}-EDTA stimulate PagP activity *in vitro*. These findings suggest that PagP remains dormant in outer membranes until Mg^{2+} limitation promotes the migration of phospholipids into the outer leaflet.

Biological membranes are built from diverse lipids and proteins. Much information has been gained on the incorporation of proteins into membranes, but the trafficking of lipids during membrane biogenesis is poorly understood (for recent reviews see Refs. 1 and 2). Lipid biosynthesis is restricted primarily to the cytosolic leaflets of either the endoplasmic reticulum membrane in eukaryotic cells or the inner (cytoplasmic) membrane in bacteria. Membrane biogenesis thus depends on distinct lipid trafficking events including lateral diffusion, translocation between opposite leaflets (known also as flip-flop), and wholesale transport to distinct cellular compartments. Both lipid translocation and lipid transport are dependent on membrane proteins that may or may not utilize cellular energy (2). Lipid transport in eukaryotes proceeds through the budding and fusion of vesicles (3) or, less frequently, through sites of membrane contact (4, 5). Although vesicles can bud off from bacterial outer membranes (6, 7), the peptidoglycan or murein layer is thought to prevent vesicle transport from bacterial cytoplasmic membranes. Possible roles for membrane contact sites or lipid transfer proteins in Gram-negative bacterial outer membrane biogenesis are subjects of current debate (1).

Lipid trafficking during biogenesis of the bacterial outer membrane begins with the biosynthesis of phospholipids and lipopolysaccharide (LPS)[1] in the inner membrane (1, 8). Passive translocation of phospholipids to the external leaflet of the inner membrane depends on the transmembrane α-helical segments of certain integral membrane proteins (9). An essential ATP-binding cassette transporter, or traffic-ATPase, MsbA is believed to translocate LPS across the inner membrane (10–14). The precise mechanism by which lipids are then transported to the outer membrane is unknown, but conditional mutants defective in MsbA have been shown recently to accumulate both phospholipids and LPS in the inner membrane (11). It has been known for over 25 years that phospholipids rapidly exchange between the inner and outer membranes, whereas the transport of LPS is unidirectional (15). Additionally, the organization of lipids in the outer membrane is highly asymmetric, with LPS located exclusively in the extracellular outer leaflet and phospholipids normally restricted to the periplasmic inner leaflet (16). The asymmetric lipid organization of the outer membrane depends on the presence of divalent cations, particularly Mg^{2+} and Ca^{2+}, which bridge negatively charged phosphate groups in the LPS (17, 18). Removal of divalent cations with chelating agents such as EDTA can strip a fraction of LPS from the cell surface (19). A large body of evidence indicates that EDTA promotes the translocation of phospholipids to the outer membrane outer leaflet (20, 21). Consequently, phospholipids lost from the inner leaflet during EDTA treatment are likely replenished by lipid transport from the cytoplasmic membrane.

PagP is an outer membrane enzyme that transfers a palmi-

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[1] The abbreviations used are: LPS, lipopolysaccharide; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; PBS, phosphate buffered saline.

tate chain from the *sn*-1 position of a phospholipid to position 2 of the lipid A (endotoxin) component of LPS (22). Lipid A is built in the cytosolic leaflet of the inner membrane from a β-1′,6-linked disaccharide of glucosamine that is acylated with (*R*)-3-hydroxymyristate at the 2-, 3-, 2′-, and 3′-positions and is phosphorylated at the 1- and 4′-positions. Two units of 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) are attached to the 6′-position, followed by the formation of acyloxyacyl linkages with laurate and myristate chains at the 2′- and 3′-positions, respectively (8). The resultant hexa-acylated Kdo_2-lipid A or Re endotoxin (Fig. 1) is the simplest chemotype of LPS that can be transported to the outer membrane, although the structure is normally further glycosylated and phosphorylated to complete the core oligosaccharide before MsbA translocates the complex to the periplasmic leaflet of the inner membrane. The antigenic O-polysaccharide is usually attached in the periplasmic space to the distal sugar of the core oligosaccharide before transport to the outer membrane, but most laboratory strains of *E. coli* K12 do not synthesize the O-antigen.

During infection, endotoxin is sensed by the immune system and can elicit a cascade of cytokine production that may lead to septic shock as a consequence of massive inflammation (23). However, palmitoylated lipid A functions as an endotoxin antagonist in human cell lines by interfering with the toll-like receptor 4 inflammatory signaling pathway (24–26) and also provides bacterial resistance to vertebrate cationic antimicrobial peptides (27). PagP promotes infections of the mammalian respiratory tract by providing bacterial resistance to host immune defenses (28, 29), including antibody-mediated complement lysis (30). The *pagP* gene is regulated by virulence-associated signal transduction pathways including *Salmonella* PhoP/PhoQ (27), which senses Mg^{2+}-limited growth conditions encountered during infection (31), *Bordetella* BvgA/BvgS (29), and *Escherichia coli* EvgA/EvgS (32). Lipid A palmitoylation appears to be a regulated process in other pathogens of humans (33), insects (34), and plants (35). We now provide evidence that phospholipid trafficking to the outer membrane outer leaflet controls PagP-catalyzed acylation of endotoxin. These findings can be rationalized with the structure and dynamics of the PagP enzyme, which has its active site located in the outer leaflet of the outer membrane (36–38).

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ was purchased from PerkinElmer Life Sciences. Antibiotics, *O*-nitrophenyl-β-D-galactoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Sigma. Pyridine, methanol, and 88% formic acid were obtained from Mallinckrodt. Chloroform was purchased from EM Science. Glass-backed Silica Gel 60 TLC plates were from Merck. The QIAprep spin miniprep, Qiaquick PCR purification, and QIAEX II gel extraction kits were obtained from Qiagen. The Easy-DNA genomic DNA isolation kit and Taq polymerase were from Invitrogen. *Pfu* and *Pfu* turbo polymerases, and supercompetent *E. coli* XL1-Blue were obtained from Stratagene. Restriction endonucleases, T4 DNA ligase, and dNTP were obtained from Fermentas. All other materials were obtained from commercial sources.

Bacterial Strains, Plasmids, Phage, and Growth Conditions—The bacterial strains, plasmids, and phage used in this study are described in Tables I and II. *E. coli* strains were kindly provided by Drs. Eduardo Groisman (FS1000), Carey Waldburger (CSH26 and CSH26ΔQ), and Christian Raetz (W3110 and WD2). Plasmid pMAK705 was provided by Dr. Sidney Kushner, whereas plasmid pRS551 and bacteriophage λRS45 were provided by Dr. Robert Simons. Cells were generally grown at 37 °C in LB broth (39) or in N-minimal medium (40). Antibiotics were added when necessary at final concentrations of 12 μg/ml for tetracycline, 20 μg/ml for chloramphenicol, 100 μg/ml for ampicillin, 100 μg/ml for streptomycin, and 40 μg/ml for kanamycin, unless indicated otherwise. Single colonies were generally inoculated from plates into 5 ml of liquid medium and grown at 37 °C overnight to stationary phase. A 1% inoculum was then subcultured into the same medium and allowed to resume growth at 37 °C. Cultures were adjusted with EDTA using a stock solution of 500 mM EDTA, pH 8.0, which had been sterilized by using a 0.2-μm filter. Standard procedures for the cultivation of bacteriophage were performed according to Silhavy *et al.* (41).

DNA Manipulations—Restriction enzyme digestions, ligations, transformations, and DNA electrophoresis were performed according to Sambrook *et al.* (42). The oligonucleotide primers used for DNA sequencing and PCR gene amplification were manufactured by Invitrogen and are described in Table III. Purification of plasmids, PCR products, and restriction fragments was performed with the QIAprep, QIAquick, and QIAEX II kits, respectively, according to the manufacturer's instructions (Qiagen). Genomic DNA was purified using the Easy-DNA kit (Invitrogen). DNA sequencing was performed at the ACGT Corp. sequencing facility (Toronto, Ontario, Canada). PCR gene amplification was performed with 2.5 units of *Pfu* polymerase in a volume of 50 μl of the supplied buffer with 100 ng of template DNA, 150 ng of the appropriate primers (Table III), and 10 μM dNTPs. After initial denaturation for 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at the appropriate annealing temperature, and 2 min at 72 °C were performed and then followed by 10 min at 72 °C. Inverse PCR (43) was performed with 2.5 units of *Pfu* Turbo polymerase in a volume of 50 μl of the supplied buffer with 5 ng of pACPagP as template DNA, 200 ng of the appropriate primers phosphorylated at the 5′-ends (Table III), and 10 μM dNTPs. After initial denaturation for 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 60 °C, and 12 min at 72 °C were performed and then followed by 10 min at 72 °C. The PCR product was blunt end-ligated by using T4 DNA ligase. Cloned PCR products were subjected to double strand DNA sequencing to confirm the absence of any spurious mutations.

Plasmid pCrcHB was constructed by cloning the 2160-bp HindIII/BglII fragment carrying *E. coli pagP*(*crcA*)*cspEcrcB* from plasmid pKH1 into the isopropyl 1-thio-β-D-galactopyranoside-inducible *tac* promoter expression vector pMS119HE (Table II), which was opened by HindIII/BamHI digestion. The *Rtem* β-lactamase gene from pUC19 was amplified by using the primers RTEM5NcoI and RTEM3SphI (Table III) at an annealing temperature of 50 °C, and cloned in the reverse orientation into the *pagP* gene of pCrcHB by NcoI/SphI digestion to create pPagAp. The disrupted *pagP*::*amp* allele in pPagAp was cloned into pMAK705 (Table II) by HindIII/SstI digestion to create pPagAp705. Replacement of the chromosomal *pagP* gene in *E. coli* MC1061 with the *pagP*::*amp* allele in pPagAp705 was performed by allelic exchange as described by Hamilton *et al.* (44). Allelic exchange was verified by PCR using genomic DNA template and the primers pagP1 and pagP2 (Table III) at an annealing temperature of 52 °C. The PCR product was sequenced using the same primers to verify the presence of the expected *Rtem* ligation junctions.

The *pagP* gene under control of its endogenous promoter was amplified from pCrcHD, using the primers HD5HindIII and HD3BamHI (Table III) at an annealing temperature of 52 °C, and cloned into pACYC184 (Table II) by HindIII/BamHI digestion to create pACPagP. The *pagP* promoter was amplified from pCrcHB, using the primers HB5EcoRI and HB3BamHI (Table III) at an annealing temperature of 46 °C with 200 μM dNTPs, and cloned in pRS551 (Table II) by EcoRI/BamHI digestion to create pPagP551. Fusion junctions were verified by sequencing using the primers opf1 and ofp2 (Table III). Recombination of pPagP551 with λRS45 (Table II) to create λRS45pagP551, and integration of λRS45pagP551 into the chromosomal *attB* locus, was performed using procedures described by Hand and Silhavy (45). Verification of single copy prophage integration was achieved by PCR using the procedure of Powell *et al.* (46), with Taq polymerase and the primers attB, attP, and int at 500 nM (Table III). β-Galactosidase activity was measured by the procedure of Miller (39), using cells that were harvested and washed with phosphate-buffered saline (PBS) (42) to prevent interference from EDTA in the assay.

Analysis of Lipid A by TLC—Analysis of lipid A released by mild acid hydrolysis from $^{32}P_i$-labeled cells was adapted from Zhou *et al.* (47). An overnight culture grown at 37 °C was diluted 100-fold into 5 ml of LB broth containing appropriate antibiotics and 5 μCi/ml $^{32}P_i$ and was allowed to grow at 37 °C for 3 h, unless indicated otherwise. The ^{32}P-labeled cells were harvested by centrifugation and washed once with 5 ml of PBS. The pellet was resuspended in 0.8 ml of PBS and converted into a single-phase Bligh/Dyer mixture (48) by adding 2 ml of methanol and 1 ml of chloroform. After 10 min of incubation at room temperature, the insoluble material was collected by centrifugation in a clinical centrifuge. The pellet was washed once with 5 ml of a fresh single-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (1:2:0.8, v/v). This pellet was then dispersed in 1.8 ml of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS, with sonic irradiation in a bath apparatus. The mixture was incubated at 100 °C for 30 min to cleave the ketosidic linkage between Kdo and the distal glucosamine sugar of lipid A. After cooling, the boiled mixture was converted to a

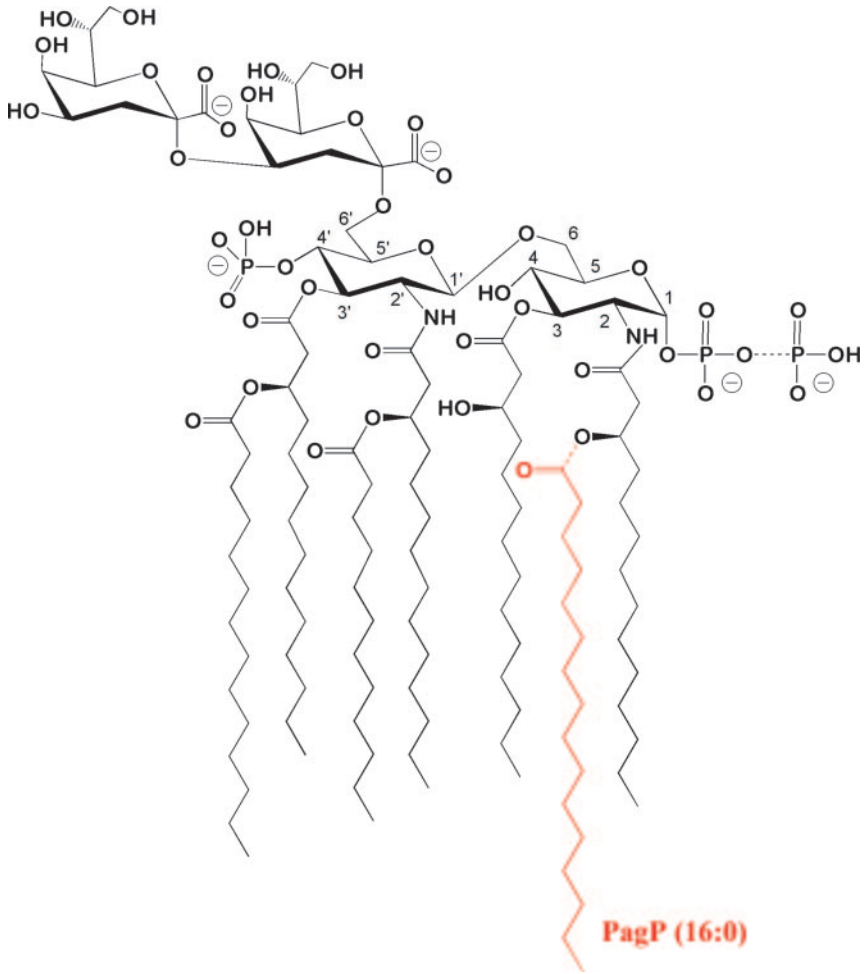

FIG. 1. **Structure of Kdo_2-lipid A (Re endotoxin) from *E. coli*.** Lipid A is built from a disaccharide of glucosamine that is acylated with (*R*)-3-hydroxymyristate chains at the 2-, 3-, 2′-, and 3′-positions, and is phosphorylated at the 1- and 4′-positions. Roughly one-third of lipid A from *E. coli* contains a diphosphate moiety at the 1-position (*dashed line*). Two Kdo sugars are attached to the 6′-position, followed by the formation of acyloxyacyl linkages with laurate and myristate chains at the 2′- and 3′-positions, respectively. A regulated proportion of lipid A in *E. coli* contains a palmitate chain (16:0), which is added to the 2-position (*red*). The phospholipid/lipid A palmitoyltransferase PagP is the only enzyme of lipid A biosynthesis in *E. coli* that is localized in the outer membrane.

two-phase Bligh/Dyer mixture by adding 2 ml of chloroform and 2 ml of methanol. Partitioning was made by centrifugation, and the lower phase material was collected and washed once with 4 ml of the upper phase derived from a fresh neutral two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). The lower phase lipid A sample was collected and dried under a stream of nitrogen gas. The lipid A sample was dissolved in 100 μl of chloroform/methanol (4:1, v/v), and an ~1000 cpm portion of the sample was applied to the origin of a Silica Gel 60 TLC plate. TLC was conducted in a developing tank in the solvent of chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). The plate was dried and visualized with a PhosphorImager (Amersham Biosciences).

Lipid A was similarly analyzed in the culture medium, following treatment of the cells with or without EDTA, with some modifications from the above procedure. Ten μCi/ml $^{32}P_i$ was added to 5 ml of LB, and the subcultured bacteria were allowed to grow for 130 min. The ^{32}P-labeled cells were harvested by centrifugation at room temperature and washed once with 5 ml of LB medium prewarmed at 37 °C. The pellet was resuspended in 5 ml of prewarmed LB medium and allowed to grow for another 20 min before a 5-min treatment with 25 mM EDTA. The culture was centrifuged, and lipid A in the cell pellet was analyzed as described above. First, the upper 3.24 ml of the supernatant was carefully removed with a pipette and transferred into a fresh tube, which was centrifuged again to remove any remaining traces of bacteria. A 0.36-ml portion of 125 mM sodium acetate, pH 4.5, containing 10% SDS was added to the culture medium and incubated at 100 °C for 30 min. After cooling, the boiled mixture was converted to a two-phase Bligh/Dyer mixture by adding 4 ml of chloroform and 4 ml of methanol. The lower phase was washed once with 8 ml of fresh upper phase and dried under a stream of nitrogen gas. The sample was analyzed by TLC as described above to verify that the radioactive material was represented by lipid A.

RESULTS

EDTA Induces Lipid A Palmitoylation—The addition of a palmitate chain to lipid A is one of several lipid A modifications that are controlled by PhoP/PhoQ in *Salmonella* (49). Lipid A modifications are not normally expressed in *E. coli*, but palmitate, phosphoethanolamine, and 4-amino-4-deoxy-L-arabinose addition can be induced in a PhoP/PhoQ-independent manner by treatment of cells with ammonium metavanadate (47). We reasoned that EDTA may specifically activate PagP, because it is the only enzyme of lipid A biosynthesis localized in the outer membrane of *E. coli* (22). The ability to induce palmitoylation independently of other lipid A modifications would greatly simplify our analysis. Therefore, we utilized a mild acid hydrolysis procedure to isolate lipid A from *E. coli* cultures following a brief 5-min treatment with EDTA in LB medium. In these experiments, LPS is recovered from cells, and the lipid A is released by pH 4.5 hydrolysis at 100 °C in SDS, which cleaves the Kdo lipid A linkage but does not disturb the acyl chains (50, 51). Two-thirds of lipid A is recovered from *E. coli* as the 1,4′-bisphosphate, whereas the remaining third contains a diphosphate group at the 1-position (10) (Fig. 1). The pH 4.5 hydrolysis also generates a small amount of lipid A 4′-monophosphate (47). The lipid A derivatives that were seen in EDTA-treated *E. coli* included additional hydrophobic species, consistent with the incorporation of a seventh acyl chain (Fig. 2). The major species that migrates above the lipid A 1,4′-bisphosphate corresponds with EV1, a lipid A derivative that was shown to be a hepta-acylated 1,4′-bisphosphate bearing a palmitoyl group at the 2-position (47). Inactivation of the chromosomal *pagP* gene by replacement with a disrupted *pagP*::*amp* allele (Tables I, II, and III) eliminates the EDTA-induced hepta-acylated lipid A species. These observations demonstrate that EDTA treatment specifically induces palmitoylation of lipid A by PagP *in vivo*.

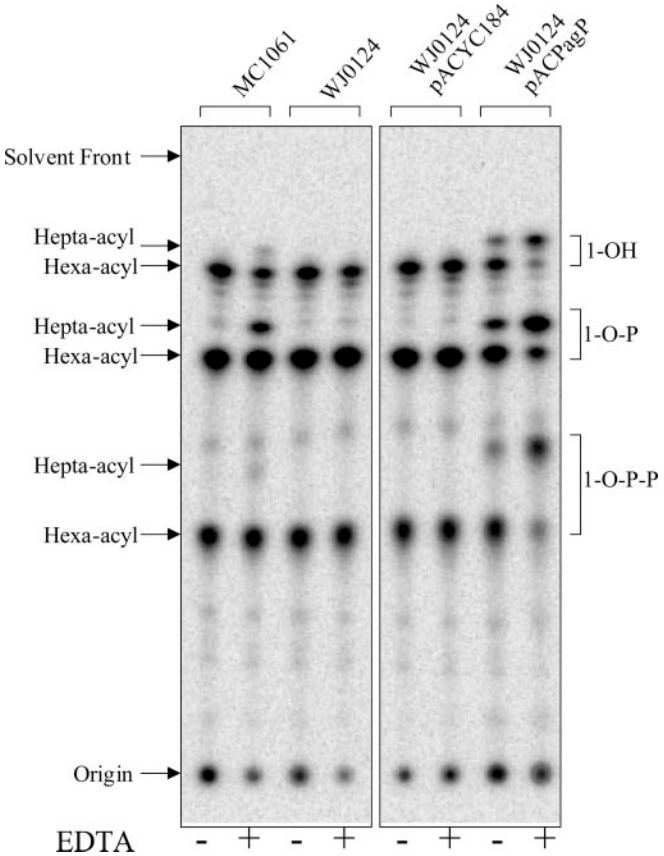

FIG. 2. **Lipid A palmitoylation is induced *in vivo* by exposure of cells to EDTA and by overexpression of PagP.** Lipid A was labeled with $^{32}P_i$ and isolated from cells by mild acid hydrolysis (47). Five-ml cultures of *E. coli* MC1061 (*wild type*) or WJ0124 (*pagP*::*amp*), and WJ0124 transformed with pACYC184 or pACPagP, were grown for 150 min and adjusted with or without 25 mM EDTA for an additional 5 min. The lipid A isolates were separated by TLC and visualized with a PhosphorImager. The three species of lipid A that were identified previously by mass spectrometry (47) are indicated to the *right* of the figure and include the lipid A 1,4′-bisphosphate (*1-O-P*), the 1-pyrophosphate (*1-O-P-P*), and the 4′-monophosphate (*1-OH*). The hexa- and hepta-acylated derivatives of each lipid A species are indicated to the *left* of the figure.

Less than 5% of *E. coli* lipid A contains palmitate in the absence of EDTA treatment (Fig. 2), suggesting that the activity of the endogenous enzyme is restricted to a low level. Roughly 20% of lipid A is modified by palmitate in *Salmonella* (52, 53), consistent with reports that PagP activity is 5–10-fold greater in membranes of *Salmonella* than *E. coli* (22). Our results indicate that EDTA treatment increases lipid A palmitoylation from roughly 20 to 50% in *Salmonella enterica* serovar Typhimurium ATCC 14028.[2] We cloned the *E. coli pagP* gene under control of its endogenous promoter into plasmid pACYC184 (Table II), which provides a gene dosage of around 18 copies per *E. coli* chromosomal equivalent during exponential growth (54). In the absence of EDTA treatment, roughly 20% of lipid A was palmitoylated in the *pagP* mutant WJ0124 that harbored plasmid pACPagP (Fig. 2). A pronounced increase in lipid A palmitoylation was then observed following EDTA treatment, resulting in palmitoylation of nearly 90% of lipid A (Fig. 2). We reasoned that the added effects of EDTA treatment and elevated *pagP* dosage on lipid A palmitoylation could be indicating a role for EDTA in the induction of *pagP* gene expression.

[2] W. Jia, A. El Zoeiby, E. I. Lo, and R. E. Bishop, unpublished observations.

EDTA Does Not Rapidly Induce pagP Gene Expression—We quantified the amount of lipid A palmitoylation observed after EDTA treatment in wild-type *E. coli* and a *phoP*::*kan* mutant (Table I). After a 20-min incubation in EDTA at 10, 25, and 50 mM, roughly 3-fold less lipid A palmitoylation was observed in the *phoP*::*kan* mutant, which still exhibited significant residual palmitoylation compared with the *pagP*::*amp* strain (Fig. 3). To determine whether these observations could be accounted for by differences in *pagP* gene expression, we monitored β-galactosidase activity from a transcriptional fusion between the *pagP* promoter and a *lacZ* reporter gene (Table II). The *pagP*(*crcA*) messenger RNA is weakly expressed in *E. coli* and is undetectable under normal growth conditions, but the transcriptional start site has been mapped to 31 bp upstream from the initiating methionine codon in a constitutively active *evgS1* mutant known to enhance expression of many PhoP/PhoQ-regulated genes (32). The *pagP*::*lacZ551* fusion in plasmid pRS551 was recombined into phage λRS45 and introduced into the chromosome as a single copy lysogen in both wild-type *E. coli* CSH26 and a Δ*phoQ* mutant CSH26ΔQ (Table I). Bacteria were cultured in N-minimal medium (40) supplemented with Mg^{2+} at 20 μM or 10 mM. A 4-fold increase in β-galactosidase activity was observed in the wild-type *pagP*::*lacZ551* strain grown in 20 μM Mg^{2+}, which was absent from the Δ*phoQ* mutant (Fig. 4). These data indicate that *E. coli pagP* is indeed induced during Mg^{2+}-limited growth by the PhoP/PhoQ two-component signal transduction pathway.

Next, we determined lipid A palmitoylation and β-galactosidase expression in parallel using both the wild-type and Δ*phoQ* mutant *pagP*::*lacZ551* fusion strains under identical growth conditions. These experiments were performed in LB medium in the presence or absence of 25 mM EDTA over the course of 1 h (Fig. 5). The optical density of growing cultures stops increasing and remains stable after EDTA treatment, indicating that cells do not undergo lysis under these conditions. No PhoP/PhoQ-dependent lipid A modifications other than palmitoylation were detected. The 3-fold decrease in lipid A palmitoylation seen in the *phoP*::*kan* strain (Fig. 3) is also seen in the Δ*phoQ* strain and can be accounted for by a 3-fold decrease in basal β-galactosidase expression driven by the *pagP* promoter (Fig. 5). The higher basal expression in the wild-type strain is consistent with previous reports (31) that LB medium is sufficiently Mg^{2+}-limited to activate *phoPQ*-dependent gene expression. EDTA treatment provides a modest 2-fold activation of β-galactosidase expression, but this occurs after a delay of more than 10 min, which may be partly accounted for by the findings that prolonged EDTA treatment of *E. coli* inhibits RNA synthesis and gradually compromises cell viability (55). Lipid A palmitoylation occurs instantaneously following EDTA treatment and is complete after less than 2 min (Fig. 5). The absence of a 2-fold increase in lipid A palmitoylation after 10 min may reflect delays not needed for the cytoplasmic β-galactosidase, but required to target PagP to the EDTA-permeabilized outer membrane. The asynchronous kinetics of *pagP* gene expression and lipid A palmitoylation raised the possibility that the latter may be induced by EDTA independently of *de novo* protein synthesis.

EDTA-induced Lipid A Palmitoylation Is Independent of de Novo Protein Synthesis—We monitored EDTA-induced lipid A palmitoylation in the presence and absence of the bacteriostatic protein synthesis inhibitor chloramphenicol. The minimal inhibitory concentration for chloramphenicol against wild-type *E. coli* strains is roughly 8 μg/ml (56). However, to achieve complete protein synthesis inhibition, much higher concentrations (170 μg/ml) are typically utilized (42). We found that the optical density of growing wild-type *E. coli* strains ceased to

TABLE I
Bacterial strains used in this work

E. coli	Genotype	Source/Ref.
XL1-Blue	F$^-$, λ$^-$, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, {F′ *proAB*, *lacI*^{q}*ZΔ M15*, Tn*10*(Tet^r)}^{c}	Stratagene
MC1061	F$^-$, λ$^-$, *araD139*, Δ(*ara-leu)7697*, Δ(*lac)X74*, *galU*, *galK*, *hsdR2* (r_K-m_K+), *mcrB1*, *rpsL*	77
WJ0124	MC1061, *pagP*::*amp*	This study
FS1000	MC1061, *phoP*::*kan*	78
CSH26	F$^-$, λ$^-$, *ara*, Δ(*lacpro*), *thi*	79
CSH26ΔQ	CSH26, Δ*phoQ*	79
W3110	F$^-$, λ$^-$	11
WD2	W3110, *msbA*^{A270T}	11

TABLE II
Bacterial plasmids and phage used in this work

Plasmid/phage	Description	Source/Ref.
Plasmid		
pKH1	pBR322-derived plasmid carrying the 2200-bp HindIII/EcoRI locus of *E. coli pagPcspEcrcB* (Ap^R)	80
pMS119HE	IPTG^a-inducible *tac* promoter expression vector (Ap^R)	81
pCrcHB	2160-bp HindIII/BglII locus from pKH1 cloned into HindIII/BamHI-digested pMS119HE (Ap^R)	This study
pCrcHD	1100-bp HindIII/DraI *pagP* fragment of pKH1 cloned into HindIII/SmaI-digested pMS119HE (Ap^R)	22
pMAK705	Low copy vector with temperature-sensitive replicon for allelic replacement (Chl^R)	44
pUC19	Template for amplification of *Rtem* β-lactamase gene (Ap^R)	82
pPagAp	*Rtem* PCR product cloned into SphI-NcoI-digested pCrcHB to create disrupted *pagP*::*amp* allele (Ap^R)	This study
pPagAp705	*pagP*::*amp* allele from pPagAp cloned by HindIII-SstI digestion into pMAK705 (Chl^R, Ap^R)	This study
pRS551	*lacZ* transcriptional fusion plasmid (Kan^R)	83
pPagP551	435-bp *pagP* promoter PCR product from pCrcHB cloned as *pagP*::*lacZ* transcriptional fusion in pRS551 by EcoRI/BamHI digestion (Kan^R)	This study
pACYC184	Low copy cloning vector (Chl^R, Tet^R)	54
pACPagP	990-bp *pagP* PCR product from pCrcHD cloned into pACYC184 by HindIII/BamHI digestion (Chl^R)	This study
pACPagPΔ29–33	Internal pACPagP deletion by primers 30R and 33F	This study
pACPagPΔ30–36	Internal pACPagP deletion by primers 30R and 36F	This study
pACPagPΔ30–39	Internal pACPagP deletion by primers 30R and 39F	This study
pACPagPΔ48–58	Internal pACPagP deletion by primers 48R and 58F	This study
Phage λ		
λRS45	λ phage designed to recombine with *lacZ* fusion plasmids for the generation of single copy lysogens	83
λRS45pagP551	λRS45 recombined with pPagP551 to generate the λ *pagP*::*lacZ*551 allele (Kan^R)	This study

[a] IPTG is isopropyl 1-thio-β-d-galactopyranoside.

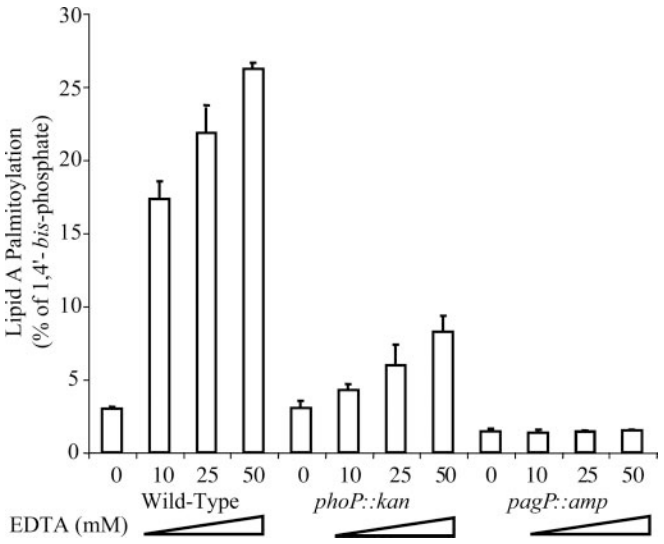

FIG. 3. **Quantification of lipid A palmitoylation as a percentage of the lipid A 1,4′-bisphosphate species.** Lipid A was labeled with $^{32}P_i$ and isolated from cells by mild acid hydrolysis (47). Five-ml cultures of *E. coli* MC1061 (*wild type*), FS1000 (*phoP*::*kan*), and WJ0124 (*pagP*::*amp*) were grown for 3 h and either harvested or adjusted with the indicated amounts of EDTA for an additional 20 min before being harvested. The lipid A isolates were separated by TLC and then visualized and quantified with a PhosphorImager.

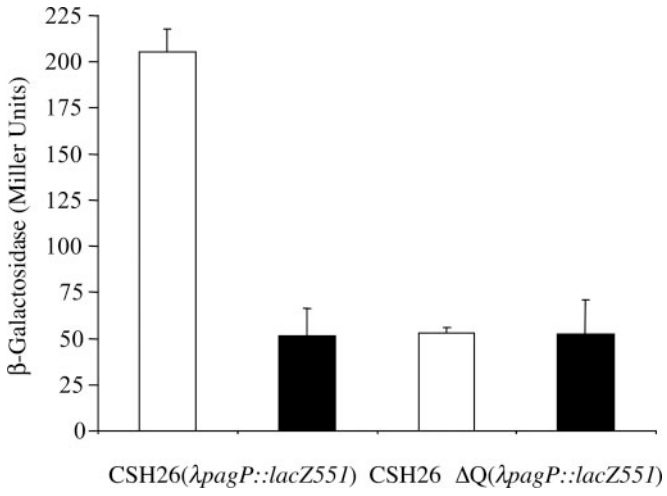

FIG. 4. **β-Galactosidase activity from a *pagP*::*lacZ* transcriptional fusion in single copy lysogens.** The *E. coli pagP* promoter was cloned in front of the *lacZ* gene in plasmid pRS551 and recombined into bacteriophage λRS45. The λ*pagP*::*lacZ551* reporter construct was moved in single copy onto the chromosome of wild-type *E. coli* CSH26 and a Δ*phoQ* mutant CSH26ΔQ. Cells were grown in N-minimal medium supplemented with 20 μM Mg^{2+} (*open bars*) or 10 mM Mg^{2+} (*filled bars*) for 6 h and 40 min until A_{260} = 0.28–0.7, and β-galactosidase activity was measured according to Miller (39).

increase immediately upon treatment with 170 μg/ml chloramphenicol, but the viable counts over the course of the treatment were unaffected. By staggering two wild-type *E. coli* cultures by 1 h and treating the first one with chloramphenicol for 1 h, the subsequent effects of EDTA treatment for 5 min could be observed at the same cell densities (Fig. 6). The results clearly show that EDTA-induced lipid A palmitoylation occurs independently of treatment with 170 μg/ml chloramphenicol. The increase in β-galactosidase activity seen in *E. coli* CSH26(λ*pagP*::*lacZ551*) over the course of a 1-h EDTA treatment (Fig. 5) was not observed in the presence of 170 μg/ml chloramphenicol, indicating that protein synthesis is inhibited under these conditions.[2]

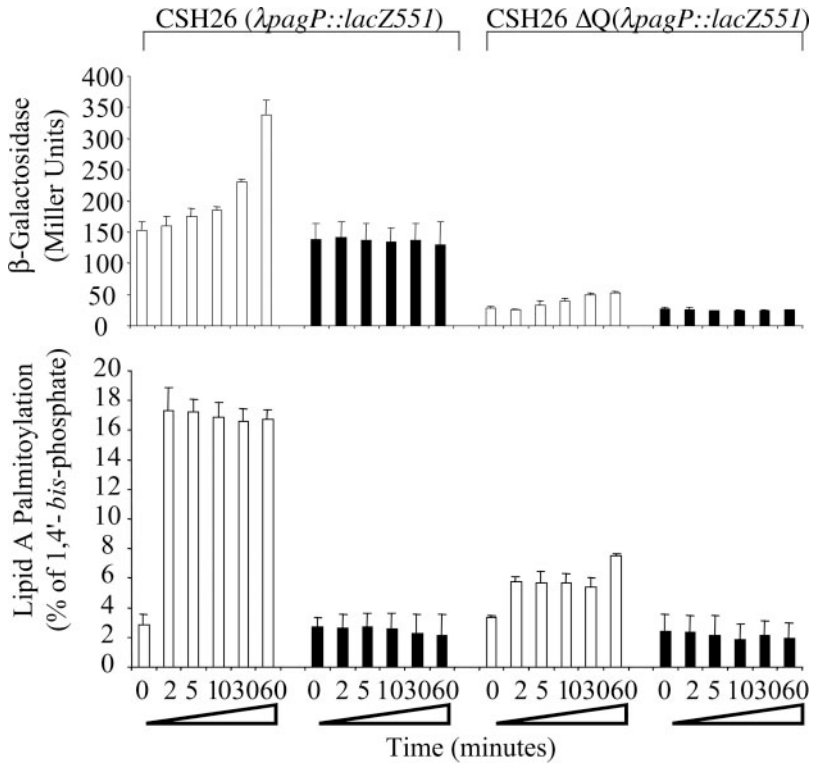

FIG. 5. **EDTA induction kinetics of *pagP* gene expression and lipid A palmitoylation.** Wild-type *E. coli* CSH26 and the Δ*phoQ* mutant CSH26ΔQ carrying the λ*pagP*::*lacZ551* reporter construct in single copy on the chromosome were grown in LB medium for 150 min. Cultures supplemented with 25 mM EDTA for an additional hour (*open bars*) were started 1 h earlier than those without EDTA (*filled bars*). β-Galactosidase activity was measured according to Miller (39) (*top panel*), and lipid A was labeled with $^{32}P_i$ and isolated from cells by mild acid hydrolysis according to Zhou *et al.* (47) (*lower panel*). The lipid A isolates were separated by TLC and visualized and quantified with a PhosphorImager.

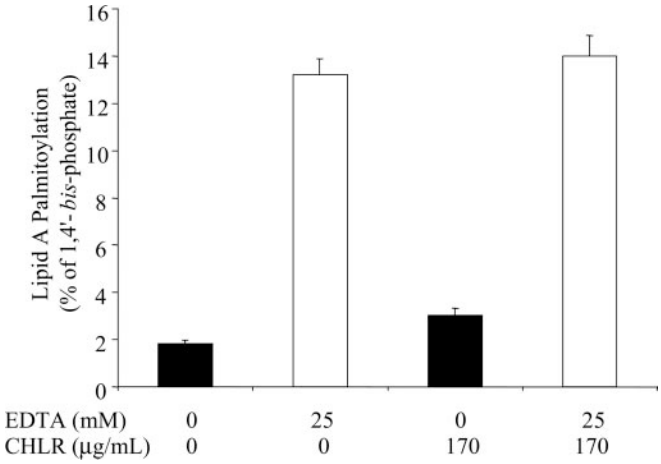

FIG. 6. **Effect of chloramphenicol on EDTA-induced lipid A palmitoylation.** Lipid A was labeled with $^{32}P_i$ and isolated from cells by mild acid hydrolysis (47). Five-ml cultures of *E. coli* MC1061 were staggered by 1 h and grown for 3 h. Cultures adjusted with 170 μg/ml chloramphenicol (*CHLR*) were incubated for an additional hour before all cultures were treated with or without 25 mM EDTA for 5 min. The lipid A isolates were separated by TLC and visualized and quantified with a PhosphorImager.

EDTA Does Not Activate PagP through Allosteric or Enrichment Mechanisms—We reasoned that PagP may be activated by EDTA through an allosteric mechanism. PagP is normally assayed *in vitro* in the presence of 10 mM EDTA (22), but we find that substitution of EDTA with either water, 10 mM $MgCl_2$, or 10 mM Mg^{2+}-EDTA does not appreciably affect lipid A palmitoylation *in vitro*. PagP assays are performed in detergent micelles, which solubilize both the enzyme and lipid substrates, but PagP normally operates in the asymmetric outer membrane bilayer *in vivo*. It has been long known that treatment of growing *E. coli* with EDTA alters the outer membrane by stripping some of the LPS molecules from the cell surface (19) and replacing them with phospholipids (20, 21). Additionally, some lipid A is normally released from *E. coli* K12 strains into LB medium through a vesiculation mechanism (7). We quantified the amount of lipid A shed into the culture medium with and without EDTA treatment in *E. coli* MC1061 (see "Experimental Procedures"). We find that the amount of lipid A released into the medium following EDTA treatment only exceeded the amount released without EDTA treatment by 2.2 ± 0.3-fold. The amount of lipid A recovered from cells in the same experiments indicated that only 11 ± 7% of the lipid A was lost from EDTA-treated cells. If the released lipid A is exclusively in the hexa-acylated form, then a 10% release would affect a minor enrichment in palmitoylated lipid A, but such an enrichment mechanism cannot account for the 6–8-fold increases routinely observed after EDTA treatment from basal levels of 2–3% lipid A palmitoylation.

Lipid Trafficking Is Required for EDTA-induced Lipid A Palmitoylation—The possibility that PagP may be activated by the translocation of phospholipids to the outer membrane outer leaflet during EDTA treatment could provide an alternative explanation. A conditional *E. coli* mutant deficient in lipid transport to the outer membrane has been described recently (11). *E. coli* WD2 (Table I) contains an A270T substitution in the transmembrane region of the ATP-binding cassette transporter MsbA and grows normally at 30 °C but accumulates 90% of cellular lipids in the inner membrane after the temperature has shifted to 44 °C. The viability of WD2 cells is not compromised over the first 45 min following growth at 44 °C, and proteins continue to be transported to the outer membrane. We grew wild-type *E. coli* and the *msbA*^{A270T} mutant at 30 °C, with or without a temperature shift to 44 °C for 45 min, followed by a 5-min treatment with 25 mM EDTA. The results of lipid A analyses indicated that no differences in the degree of lipid A palmitoylation were observed when bacteria were grown at 30 °C (Fig. 7). However, following a shift to the nonpermissive condition for lipid transport (44 °C), the extent of lipid A palmitoylation after EDTA treatment in the *msbA*^{A270T} mutant was drastically reduced in comparison to the wild-type strain.

The PagP Amphipathic Helix Does Not Promote Phospholipid Translocation—PagP is an 8-stranded antiparallel β-barrel

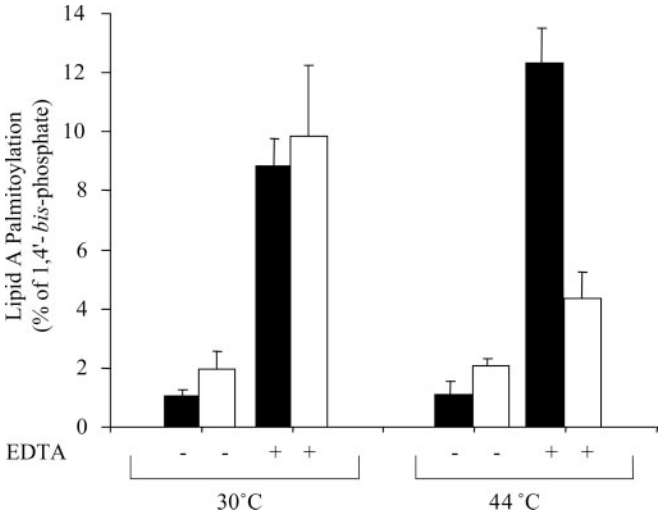

FIG. 7. **EDTA-induced lipid A palmitoylation in a conditionally defective lipid transport mutant of *E. coli*.** Lipid A was labeled with $^{32}P_i$ and isolated from cells by mild acid hydrolysis (47). Five-ml cultures of *E. coli* W3110 (*wild-type*; *filled bars*) and WD2 (*msbA*^{A270T}; *open bars*) were grown at 30 °C for 165 min and then grown an additional 45 min at either 30 or 44 °C before treatment with or without 25 mM EDTA for 5 min. The lipid A isolates were separated by TLC and visualized and quantified with a PhosphorImager.

preceded by an N-terminal amphipathic α-helix (36). Because amphipathic helices can affect outer membrane structure (57, 58), we created a series of deletion mutations in the PagP N-terminal helix in order to investigate a possible role in the translocation of the phospholipid substrate across the outer membrane. A modified inverse PCR using pACPagP as template DNA was employed for the construction of in-frame deletion mutants (Fig. 8*A*). The primers designed for inverse PCR are shown in Table III. To avoid interfering with signal peptide processing, we retained the first 3 or 4 residues of the mature N terminus. Additionally, we retained the last 5 residues at the C terminus of the helix because these residues form a hydrogen-bonded network that fixes the helix against the β-barrel and covers a polar patch that would otherwise be exposed to the lipid bilayer (38). A deletion mutation of the first β-sheet of the β-barrel was also created to be included as a control for PagP inactivation, because the disruption of the β-barrel structure should undoubtedly interfere with catalysis. The amount of lipid A palmitoylation observed before and after EDTA treatment in the wild-type and the deletion mutants (Fig. 8*B*) clearly demonstrates that the omission of most of the α-helix does not interfere with PagP activity *in vivo*, whereas the omission of the β-sheet abolishes activity. The only invariant residue in the PagP helix is Trp-17, which contributes to the inner aromatic belt and the aforementioned helix-barrel interactions. Because the bulky indole group of Trp-17 projects upward into the phospholipid-containing inner leaflet of the outer membrane, we created a W17A mutant in pACPagP, but the *E. coli* WJ0124 (*pagP*::*amp*) transformant was not compromised in its ability to palmitoylate lipid A *in vivo*.[2]

DISCUSSION

We have demonstrated that brief treatment of growing *E. coli* with EDTA can specifically induce the palmitoylation of lipid A by the outer membrane enzyme PagP. The finding that the *E. coli pagP* gene is induced under Mg^{2+}-limited growth conditions by the PhoP/PhoQ two-component signal transduction pathway suggested first that EDTA induction of lipid A palmitoylation might occur through the activation of *pagP* gene expression. However, EDTA activates *pagP* expression too slowly to account for the rapid palmitoylation of lipid A, which was ultimately shown to occur independently of *de novo* protein synthesis. A second possibility that EDTA or Mg^{2+}-EDTA stimulates PagP activity is inconsistent with measurements performed *in vitro*. Third, EDTA could simply enrich LPS that contains palmitoylated lipid A in the outer membranes of cells by selectively stripping those LPS molecules that lack palmitate in lipid A. Although EDTA is reported to strip 30–50% of LPS from the cell surface (59, 60), closer to 10% is stripped when O-antigen is absent from the LPS (61) as occurs in our derivatives of *E. coli* K12. Because we consistently found that the basal level of palmitoylated lipid A in *E. coli* is only 2–3% of the total before EDTA treatment, the vast majority of LPS would have to be stripped from the cell surface to achieve our observed levels of EDTA-induced lipid A palmitoylation by an enrichment mechanism. A fourth possibility that PagP is activated by the translocation of phospholipids to the outer membrane outer leaflet during EDTA treatment (20, 21) was supported by the observation that a conditional MsbA mutant defective in lipid transport to the outer membrane showed a significant reduction in EDTA-induced lipid A palmitoylation. A role for lipid trafficking in the control of endotoxin palmitoylation in outer membranes is strongly supported by recent investigations into PagP structure and function.

The global fold and dynamics of *E. coli* PagP in detergent micelles were recently determined by NMR spectroscopy (36). PagP is an 8-stranded antiparallel β-barrel preceded by an N-terminal amphipathic α-helix. The β-barrel is well defined in the NMR structure, whereas the extracellular loops are not. Although relaxation measurements indicated that the α-helix is held rigidly in place, its precise positioning could not be determined due to insufficient restraint information connecting the helix to the barrel. Interestingly, the continuity of hydrogen bonding between β-strands is disrupted at two positions in the upper half of the barrel by several unusually positioned proline residues (36, 37). The PagP global fold was recently verified by the crystal structure at 1.9 Å resolution (38). Although the L1 loop and helix terminus are disordered and cannot be visualized, the helix can be clearly seen to lie against the barrel. Additional features seen in the crystal structure include the locations of amino acid side chains and the visualization of bound detergent and solvent molecules. Water molecules typically line the interior of small outer membrane β-barrel proteins (62), but this only holds true in the lower half of PagP. The interior of the upper half of the PagP barrel is occupied by a single detergent molecule, which marks an acyl chain-binding pocket that determines palmitate specificity. The amino acid residues that were previously implicated in catalysis, including Ser-77, Asp-76, and His-33 (36), reside at the extracellular surface and face the barrel interior (Fig. 9). The PagP structure predicts that both the phospholipid and LPS substrates must gain access to the barrel interior from the outer leaflet, which is incompatible with the maintenance of normal outer membrane lipid asymmetry.

We propose that PagP is assembled in the outer membrane where it remains dormant when lipid asymmetry is maintained. The Mg^{2+} ions that are needed to maintain lipid asymmetry can be removed by EDTA, which promotes phospholipid migration into the outer leaflet thereby providing PagP with access to its substrate (Fig. 9). Phospholipid migration into the outer leaflet hinges on the replenishment of phospholipids in the inner leaflet by MsbA-mediated transport from the inner membrane. The resulting palmitoylation of lipid A may represent an adaptive response to Mg^{2+}-limited growth conditions, which is consistent with the fact that *pagP* gene expression is governed by the Mg^{2+}-sensing PhoP/PhoQ two-component signal transduction pathway. The delay in responding to assaults

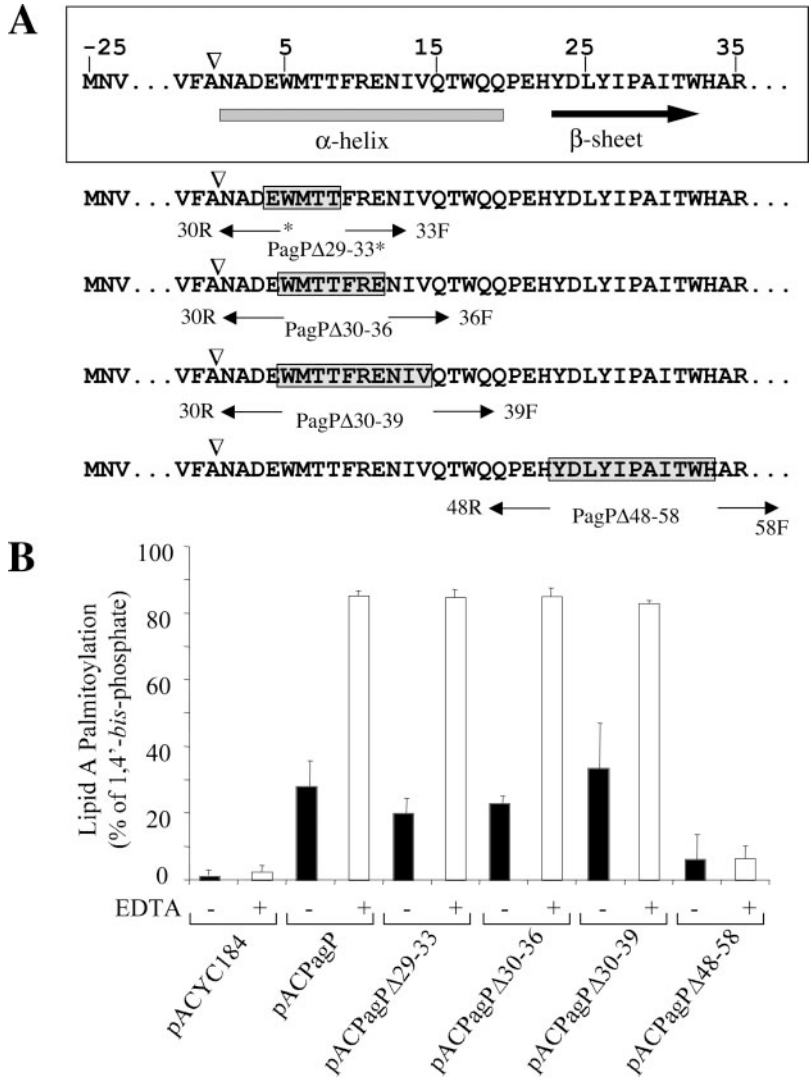

FIG. 8. **The PagP N-terminal amphipathic α-helix is dispensable for lipid A palmitoylation *in vivo*.** *A,* general strategy for the construction of pACPagP mutants using a modified inverse PCR. Blunt end ligation of the inverse PCR products leads to the desired in-frame deletions. The N-terminal 60 amino acids are identified with the signal peptidase cleavage site marked by an *inverted triangle.* The positions of the α-helix and first β-strand are also indicated. Amino acid sequences of internal deletions are shown within *shaded boxes.* The * indicates that an upstream deletion of a further 3 nucleotides, presumably due to a noncanonical ligation event, resulted in the deletion of one extra amino acid residue. *B,* lipid A was labeled with $^{32}P_i$ and isolated from cells by mild acid hydrolysis (47). Five-ml cultures of *E. coli* WJ0124 (*pagP*::*amp*), transformed with pACYC184, pACPagP, or pACPagP deletion mutants, were grown for 150 min and adjusted with or without 25 mM EDTA for an additional 5 min. The lipid A isolates were separated by TLC and visualized and quantified with a PhosphorImager.

TABLE III
Oligonucleotide primers used in this work

Name	Function	Sequence
RTEM5NcoI	PCR for pPagAp	5′ ATCCATGGAGGTGGCACTTTTCGGG 3′
RTEM3SphI	PCR for pPagAp	5′ ATATAGCATGCCGAAAACTCACGTTAAGGG 3′
PagP1	Mutation verification	5′ ACGTGAGTAAATATGTCGCT 3′
PagP2	Mutation verification	5′ GAATCCCAGACCTAAATGAA 3′
HD5HindIII	PCR for pACPagP	5′ TTCCCAAGCTTGCAAATTGATGGTGGATTG 3′
HD3BamHI	PCR for pACPagP	5′ CGGGATCCGGTACCCAAAGAAGTTACT 3′
HB5EcoRI	PCR for pPagP551	5′ ATGAATTCCATTAAGACATTGAAGTTGC 3′
HB3BamHI	PCR for pPagP551	5′ ATGGATCCAAAAATCTACTACTAGCATAG 3′
Opf1	Fusion verification	5′ TCTGTTGTTTGTCGGTGAAC 3′
Ofp2	Fusion verification	5′ AGGCGATTAAGTTGGGTAAC 3′
33F	Internal deletion	5′ TTTAGAGAAAATATTGCACAAACC 3′
36F	Internal deletion	5′ AATATTGCACAAACCTGGCAAC 3′
39F	Internal deletion	5′ CAAACCTGGCAACAGCCTG 3′
30R	Internal deletion	5′ CTCATCTGCGTTAGCAAAAAC 3′
58F	Internal deletion	5′ GCACGTTTCGCTTACGACAAA 3′
48R	Internal deletion	5′ ATGTTCAGGCTGTTGCCAGG 3′
AttB	Lysogen verification	5′ GAGGTACCAGCGCGGTTTGATC 3′
AttP	Lysogen verification	5′ TTTAATATATTGATATTTATATCATTTTACGTTTCTCGTTC 3′
Int	Lysogen verification	5′ ACTCGTCGCGAACCGCTTTC 3′

on the integrity of the outer membrane through signal transduction may necessitate an additional mechanism that responds instantaneously to changes in outer membrane lipid organization. In this way, PagP becomes exquisitely sensitive to the divalent cations that represent an Achilles' heel of outer membrane structure.

Recent studies (9) have shown that a subset of integral membrane proteins can passively promote the translocation of phospholipids across membrane bilayers. The outer membrane β-barrel protease OmpT (63) was shown to lack any propensity to promote lipid translocation in these studies. If PagP behaves similarly, then its overexpression should not result in elevated lipid A palmitoylation until lipid asymmetry is broken by EDTA. Our observations that PagP overexpression did result in in-

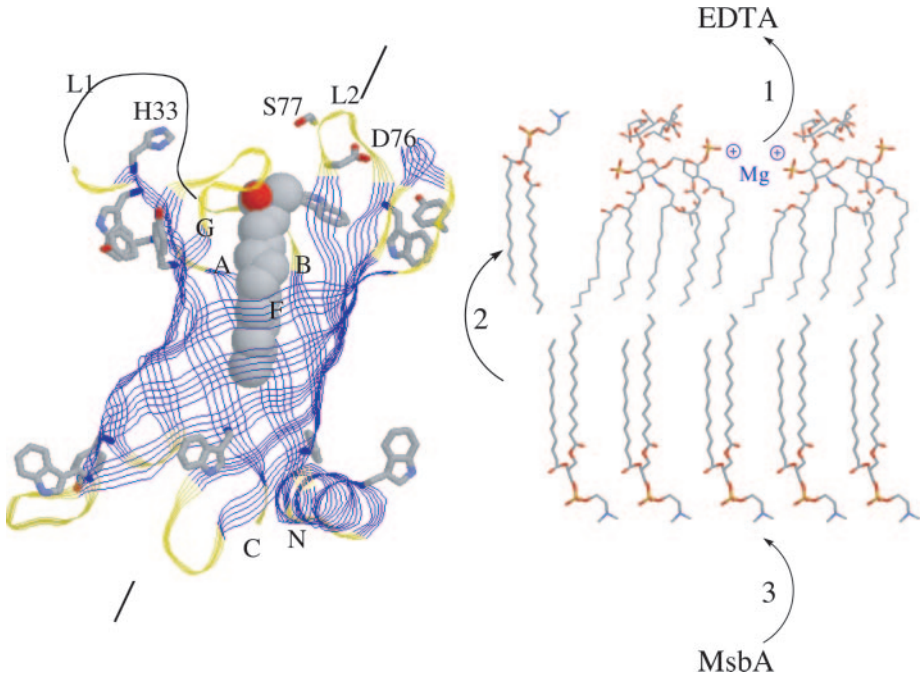

FIG. 9. **Model for lipid trafficking in the control of PagP-catalyzed endotoxin palmitoylation in outer membranes.** PagP is an 8-stranded antiparallel β-barrel preceded by an N-terminal amphipathic α-helix. A detergent molecule bound inside the β-barrel in the outer leaflet defines the hydrocarbon ruler (shown in *space filling*). Possible routes for lateral diffusion of lipid substrates to the β-barrel interior are apparent in the outer leaflet between strands A and B at the rear of the model, and between strands F and G near the front. The approximate position of the disordered extracellular loop L1 is indicated by a *curved black line*; the ordered loop L2 is also indicated. Amino acid residues implicated in catalysis, including Ser-77, Asp-76, and His-33, are located on the extracellular side facing the β-barrel interior. Tyrosine and tryptophan residues in the aromatic belts demarcate the membrane interfaces, which are only apparent when the barrel axis is tilted by 25° with respect to the plane of the membrane. The necessary steps for phospholipid trafficking to the PagP active site during EDTA treatment include the following: 1) destabilization of lateral LPS-LPS interactions by the removal of Mg^{2+}; 2) phospholipid translocation into the outer leaflet; and 3) MsbA-mediated transport of phospholipids from their source of biosynthesis in the inner membrane to the outer membrane inner leaflet.

creased lipid A palmitoylation in the absence of EDTA suggests that the enzyme possesses the means to promote phospholipid migration on its own accord. We hypothesized a role for the amphipathic α-helix in the assistance of phospholipid translocation across the outer membrane. However, our deletion mutation study ruled out such a role. Whereas all β-barrel membrane proteins of known structure have their barrel axis fixed vertical with respect to the membrane normal (62), the PagP barrel axis is uniquely tilted by 25° (38). It remains to be determined if PagP may somehow promote phospholipid translocation through a shearing action of the tilted β-strands. Once phospholipids gain access to the outer leaflet, we suggest that they enter the barrel by lateral diffusion through one of the two non-hydrogen-bonded domains between β-strands in the outer leaflet (36, 37). The hydrophobic pocket functions as a hydrocarbon ruler, which provides PagP with a means to select a palmitate chain from other acyl chains that are present in phospholipids (38). In this way, PagP is distinguished from the Ca^{2+}-dependent outer membrane phospholipase OMPLA, which exhibits a relaxed acyl chain specificity (64).

The crystal structure of OMPLA from *E. coli* reveals 12 antiparallel β-strands that fold into a transmembrane β-barrel where the central cavity is occluded by an intricate hydrogen-bonding network (65). The three active site residues, His-142, Ser-144, and Asn-156 of OMPLA are organized in a catalytic triad in the outer leaflet on the exterior of the β-barrel. The phospholipid substrates that line the inner leaflet are not normally accessible to the active site of OMPLA when outer membrane lipid asymmetry is intact. This is consistent with the findings that OMPLA usually remains dormant in the outer membrane (66). Events that disrupt lipid asymmetry when divalent cations are present, such as phage-induced lysis (67) or colicin secretion (68), can activate OMPLA, which is regulated by Ca^{2+}-dependent dimerization (65, 69). Conceivably, OMPLA and PagP may be adapted to respond to disruptions of outer membrane lipid asymmetry under Ca^{2+}-replete and Mg^{2+}-limited growth conditions, respectively. Bacterial pathogenesis can require distinct adaptive responses to Ca^{2+}-replete extracellular and Mg^{2+}-limited intracellular environments (70, 71), consistent with observations that both OMPLA and PagP are important for disease causation.

It remains to be determined precisely what physiological conditions stimulate lipid A palmitoylation by promoting phospholipid translocation to the outer membrane outer leaflet. Some antimicrobial proteins, such as bactericidal permeability-increasing protein (72) and the membrane attack complex of serum complement (73), are believed to perturb outer membrane lipid asymmetry. However, antimicrobial proteins have an avid affinity for lipid A and may sequester lipid A from the PagP active site. Although PagP activity *in vitro* conserves the pre-existing bond energy in the phospholipid donor (74) and, thus, has no exogenous energy requirement, antimicrobial proteins also disrupt vital processes in the inner membrane and likely interfere with the energetics of lipid transport on which PagP depends *in vivo*. In light of recent findings that antimicrobial peptides can activate the PhoP/PhoQ system (75), it will be interesting to learn whether the same peptides can activate PagP directly through a membrane perturbation mechanism.

Finally, EDTA treatment was originally developed to effect an increase in outer membrane permeability, which was shown to be gradually restored through a process that is dependent on energy metabolism but independent of *de novo* protein synthesis (76). The mechanism was never discovered, but it remains a distinct possibility that PagP contributes to the restoration of the permeability barrier in EDTA-treated cells. Consequently, PagP may function to both resist host immune defenses and

maintain outer membrane lipid asymmetry under Mg^{2+}-limited growth conditions.

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