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Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria

Russell E.Bishop[1,2], Henry S.Gibbons[1], Tina Guina[3], M.Stephen Trent[1], Samuel I.Miller[3] and Christian R.H.Raetz[1,4]

[1]Department of Biochemistry, Duke University Medical Center, 255 Nanaline Duke Building, Box 3711, Durham, NC 27710 and [3]Departments of Microbiology and Medicine, University of Washington, K-140 Health Sciences Building, Box 357710, Seattle, WA 98195, USA

[2]Present address: Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, 6213 Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8

[4]Corresponding author
e-mail: raetz@biochem.duke.edu

Regulated covalent modifications of lipid A are implicated in virulence of pathogenic Gram-negative bacteria. The *Salmonella typhimurium* PhoP/PhoQ-activated gene *pagP* is required both for biosynthesis of hepta-acylated lipid A species containing palmitate and for resistance to cationic anti-microbial peptides. Palmitoylated lipid A can also function as an endotoxin antagonist. We now show that *pagP* and its *Escherichia coli* homolog (*crcA*) encode an unusual enzyme of lipid A biosynthesis localized in the outer membrane. PagP transfers a palmitate residue from the *sn*-1 position of a phospholipid to the N-linked hydroxymyristate on the proximal unit of lipid A (or its precursors). PagP bearing a C-terminal His_6-tag accumulated in outer membranes during overproduction, was purified with full activity and was shown by cross-linking to behave as a homodimer. PagP is the first example of an outer membrane enzyme involved in lipid A biosynthesis. Additional *pagP* homologs are encoded in the genomes of *Yersinia* and *Bordetella* species. PagP may provide an adaptive response toward both Mg^{2+} limitation and host innate immune defenses.

Keywords: endotoxin/lipid A/outer membranes/*pagP*/palmitate

Introduction

Lipid A is the hydrophobic anchor of lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria (Raetz, 1996). The Gram-negative outer membrane has unique permeability properties because of its asymmetric bilayer architecture, with LPS lining the outer leaflet and phospholipids lining the inner leaflet (Nikaido, 1996). Lipid A is the active component of LPS endotoxin, which can promote septic shock when shed from bacteria during severe infection. Lipid A is also an essential structural component unique to the Gram-negative cell envelope and, consequently, provides a target for the design of novel anti-bacterial and anti-inflammatory agents. The major lipid A species of enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, is a β(1′-6)-linked disaccharide of glucosamine, acylated with *R*-3-hydroxymyristate at the 2, 3, 2′ and 3′ positions, and phosphorylated at the 1 and 4′ positions (Figure 1A; Raetz, 1996). The characteristic acyloxyacyl linkages of lipid A result from the addition of laurate and myristate to the hydroxyl groups of the *R*-3-hydroxymyristate moieties at the 2′ and 3′ positions, respectively (Figure 1A). Over the past 15 years, the biosynthetic enzymes that generate the constitutive lipid A species shown in Figure 1A have been identified and their corresponding structural genes cloned, primarily from *E.coli* (Raetz, 1996).

Recently, several regulated covalent modifications of lipid A have been implicated in virulence of pathogenic Gram-negative bacteria, such as *S.typhimurium* (Guo *et al*., 1997). Substitution of the phosphate groups at the 1 and 4′ positions with phosphoethanolamine and/or L-4-aminoarabinose (Figure 1B) provides resistance against polymyxin B, a lipid A-binding cationic cyclic peptide antibiotic (Vaara *et al*., 1981; Helander *et al*., 1994). In addition, modification of lipid A with palmitate in acyloxyacyl linkage at the 2 position (Figure 1B) provides resistance against cationic anti-microbial peptides (CAMPs) that are induced by the innate immune response to bacterial infections (Guo *et al*., 1998). Lipid A modified with palmitate also antagonizes LPS-mediated signal transduction in human cell lines (Tanamoto and Azumi, 2000). Lipid A modifications in *S.typhimurium* depend largely on the *phoP*/*phoQ* two-component signal transduction pathway (Groisman *et al*., 1989; Miller *et al*., 1989), which responds to the Mg^{2+}-limited growth conditions encountered during infection (Garcia-Vescovi *et al*., 1996). Furthermore, phosphoethanolamine and L-4-aminoarabinose addition to lipid A depends on the *phoP*/*phoQ*-activated genes *pmrA*/*pmrB* (Gunn *et al*., 1998), which encode a different two-component signal transduction pathway. The latter is coordinated with *phoP*/*phoQ* by a mediator known as *pmrD* (Kox *et al*., 2000). Recent studies have shown that the incorporation of palmitate into *S.typhimurium* lipid A additionally depends upon a specific *phoP*/*phoQ*-activated gene, designated *pagP* (Guo *et al*., 1998).

Modifications of lipid A with L-4-aminoarabinose, phosphoethanolamine and/or palmitate are seen in *E.coli* K-12 only when cells are treated with ammonium metavanadate (Zhou *et al*., 1999), or in polymyxin resistant mutants (Helander *et al*., 1994). These findings demonstrate that the modification enzymes are more latent (or regulated differently) in *E.coli* K-12 than in *S.typhimurium*. Lipid A modifications are not restricted

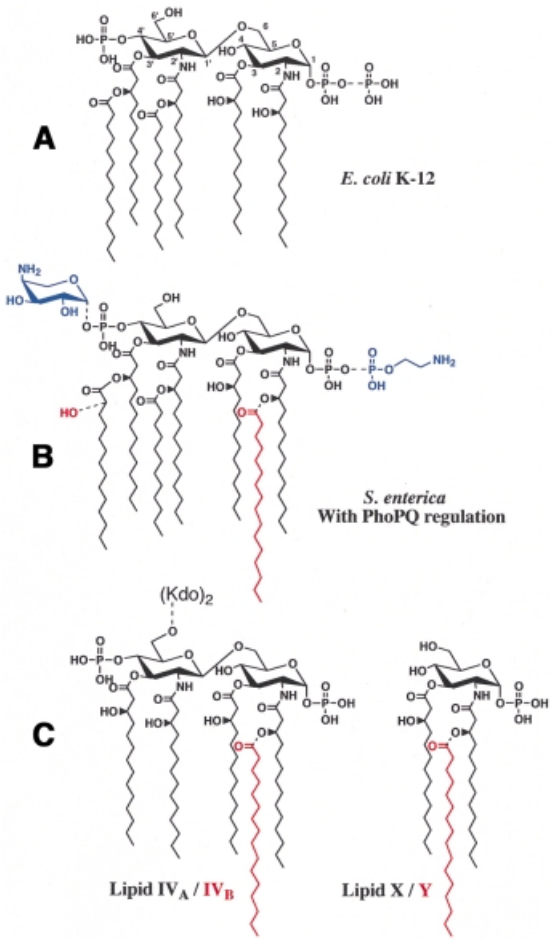

Fig. 1. Structures of lipid A species and key precursors found in *E.coli* K-12 and *S.typhimurium*. (**A**) About two-thirds of lipid A is normally recovered from *E.coli* K-12 as a hexa-acylated 1,4′-*bis*-phosphate with the remaining one-third containing a 1-pyrophosphate group (dashed bond; Zhou *et al*., 1999). (**B**) Regulated covalent lipid A modifications found in *Salmonella enterica* serovars include the L-4-aminoarabinose and phosphoethanolamine substituents (blue), and the *S*-2-OH and palmitoyl groups (red; Guo *et al*., 1997). With the exception of the *S*-2-OH group, latent enzymes for these modifications also are present in *E.coli* K-12 (Zhou *et al*., 1999), and are induced by treatment with ammonium metavanadate, or accumulate in polymyxin-resistant mutants. (**C**) Substrates utilized in this investigation include the disaccharide lipid A precursors lipid IV_A and Kdo_2–lipid IV_A (dashed bond), which can be acylated (red) by PagP to produce lipid IV_B and Kdo_2–lipid IV_B, respectively. The monosaccharide lipid X can also be acylated (red) by PagP to produce lipid Y (Brozek *et al*., 1987).

to enteric Gram-negative bacteria. In the opportunistic pathogen *Pseudomonas aeruginosa*, *phoP*/*phoQ*-dependent lipid A modifications are enhanced in bacteria isolated from cystic fibrosis patients with chronic airway infections (Ernst *et al*., 1999).

The enzymology of lipid A modification has not been explored. In 1987, an enzyme was identified in *E.coli* membranes that catalyzes the transfer of a palmitate chain from a phospholipid to a monosaccharide lipid A precursor, termed lipid X, to produce lipid Y (Brozek *et al*., 1987) (Figure 1C). The enzyme was neither purified nor the corresponding gene cloned. It was found to be very specific for palmitate at the *sn*-1 position of the phospholipid donor substrate, but non-specific for the polar head group (Brozek *et al*., 1987). Unlike other enzymes that generate acyloxyacyl moieties on lipid A (Brozek and Raetz, 1990), the palmitoyl transferase does not require thioester-containing substrates. We now demonstrate that the *Salmonella pagP* gene codes for the palmitoyl transferase first described by Brozek *et al*. (1987). Its homolog, known as *crcA* in *E.coli*, had been discovered without knowledge of the relevant biochemical function because it is one of three genes that in multiple copies confer resistance to camphor vapors (Hu *et al*., 1996). The products of the *pagP* and *crcA* genes are hereafter referred to as PagP. PagP is the first example of an enzyme of lipid A biosynthesis that is localized in the outer membrane. Homologs of *pagP* are encoded in the genomes of *Yersinia pestis*, *Bordetella pertussis*, *Bordetella bronchiseptica* and various strains of *Salmonella*.

Results

Palmitoyl transferase assays with Salmonella membranes

To determine whether or not *pagP* is required for palmitoyl transferase activity *in vitro*, we prepared membranes from *Salmonella enterica* serovar *typhimurium* strains ATCC 14028 (wild type), its PhoP-constitutive ($PhoP^c$) mutant CS022, and a *pagP* mutant (LG069) derived from CS022. Membranes were assayed for palmitoyl transferase (PagP) activity, initially using the precursors lipid X, lipid IV_A or 3-deoxy-D-*manno*-octulosonic acid $(Kdo)_2$–lipid IV_A as acyl acceptors (Figure 1C). The acylated products of the reactions were resolved by thin-layer chromatography (TLC) followed by PhosphorImager analysis (Figure 2). Wild-type *Salmonella* membranes convert lipid X to a more hydrophobic metabolite (Figure 2A), which migrates with lipid Y, as previously characterized (Brozek *et al*., 1987). Lipid IV_B (Figure 1C) and Kdo_2–lipid IV_B are also generated under these conditions (Figure 2B and C). Acylated product formation was ~5-fold higher in $PhoP^c$ membranes, and was largely eliminated in the *pagP* mutant membranes. Residual acylation detected *in vitro* with the *pagP* mutant (Figure 2) suggests that an additional lipid A-modification enzyme may be present in these extracts. However, the results of Guo *et al*. (1998) indicate that this residual activity does not contribute significantly to lipid A acylation *in vivo*. These findings are consistent with the idea that *pagP* is the structural gene for the palmitoyl transferase first described by Brozek and coworkers (Brozek *et al*., 1987). Unexpectedly, the $PhoP^c$ membranes also revealed the presence of an additional PhoP regulated enzyme that generates a more hydrophilic metabolite with each of the substrates (Figure 2). A separate investigation has shown that these hydrophilic products are made by a novel *Salmonella phoP*/*phoQ*-regulated lipid A 3-*O*-deacylase (M.S.Trent, W.Pabich, C.R.H.Raetz and S.I.Miller, unpublished data).

Outer membrane localization of PagP

In contrast to the acyl carrier protein-dependent lauroyl- and myristoyl-transferases (LpxL and LpxM, respectively) (Brozek and Raetz, 1990; Carty *et al*., 1999), PagP is dependent on acyl groups derived from phospholipids (Brozek *et al*., 1987). The unique substrate requirements of PagP do not restrict it to an inner membrane localization,

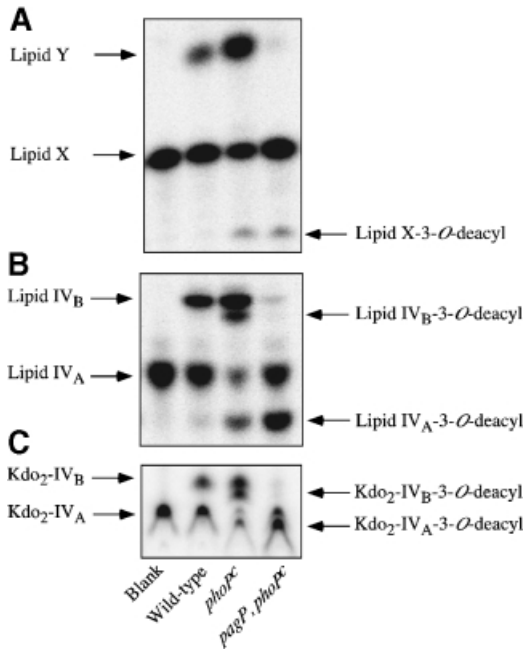

Fig. 2. Products of PagP reactions using various *Salmonella* membranes with lipid X, lipid IV_A or Kdo_2–lipid IV_A as acceptors. PagP was assayed at 30°C for 40 min using 0.5 mg/ml membranes from *S.enterica* serovar *typhimurium* ATCC 14028 (Wild-type), CS022 (*phoP*^{c}) and LG069 (*pagP*, *phoP*^{c}). Assays contain 100 mM Tris–HCl pH 8, 10 mM EDTA, 0.25% Triton X-100, 1 mM *sn*-1-(16:0)-2-(18:1cΔ^9)-PtdCho as acyl donor and 200 c.p.m./μl of the ^{32}P-labeled lipid X, lipid IV_A or Kdo_2–lipid IV_A acyl acceptors at 10 μM. Metabolites were separated by TLC and visualized by overnight exposure to a PhosphorImager screen. The solvent systems for the indicated acyl acceptors are followed by the Rf values listed in ascending order for each metabolite (identified with arrowheads). (**A**) Lipid X, chloroform:methanol:water:acetic acid (25:15:4:2 v/v). $Rf_1 = 0.42$, $Rf_2 = 0.55$, $Rf_3 = 0.71$. (**B**) Lipid IV_A, chloroform: pyridine:88% formic acid:water (50:50:16:5 v/v). $Rf_1 = 0.36$, $Rf_2 = 0.43$, $Rf_3 = 0.52$, $Rf_4 = 0.55$. (**C**) Kdo_2–lipid IV_A, chloroform:pyridine:88% formic acid/water (30:70:16:10 v/v). $Rf_1 = 0.38$, $Rf_2 = 0.40$, $Rf_3 = 0.43$, $Rf_4 = 0.45$.

which is the case for all other known membrane-bound enzymes of lipid A biosynthesis (Raetz, 1996). Therefore, protein profiles of highly purified *S.typhimurium* outer membranes were compared by 2D-PAGE as described previously (Guina *et al*., 2000). One of the PhoP-activated outer membrane protein species that was missing in the isogenic *pagP* mutant migrated at the molecular weight and pI predicted for mature PagP (18 kDa and pI 6.5; Figure 3). Corresponding protein was digested in-gel with trypsin and the resulting peptide masses were recorded by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) for peptide mass fingerprinting using the MS-Fit program of Protein Prospector (http://prospector.ucsf.edu) that was linked to the NCBI genome database (ftp://ncbi.nlm.nih.gov/blast/db/). Allowing a mass accuracy of ±0.5 Da, seven peptides matched the masses of predicted PagP tryptic fragments. Therefore, PagP is localized in the outer membrane of *S.typhimurium*.

This observation predicts that PagP activity should co-localize with outer membranes. In order to avoid interference from the *Salmonella* lipid A 3-*O*-deacylase and to exploit the established lipid A enzymology of *E.coli*, we

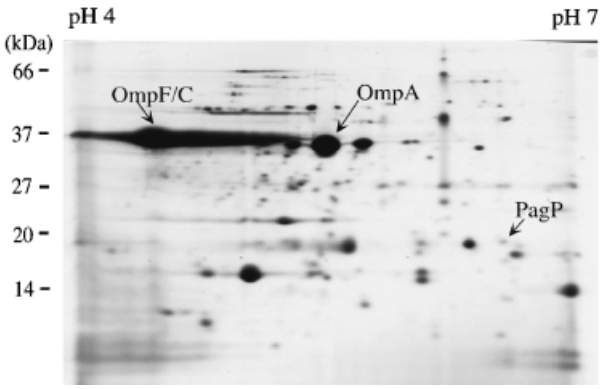

Fig. 3. *Salmonella* PagP is an outer membrane protein. Purified outer membrane proteins (350 μg) from the *S.typhimurium* PhoP-constitutive strain (CS022) were prepared and separated by 15% 2D-PAGE as described previously (Guina *et al*., 2000). An ~18-kDa protein species migrating at pI 6.5 was identified as PagP by MALDI-TOF MS tryptic peptide fingerprinting. Corresponding protein was absent from the outer membranes of the *pagP* mutant strain (LG069). The locations of PagP and the major outer membrane proteins OmpF/C and OmpA are indicated with arrowheads. The positions of molecular weight standards are indicated to the left of the gel and the linear pH gradient generated during the isoelectric focusing step is indicated above the gel.

elected to focus our investigation on *E.coli* PagP. We separated inner and outer membranes of wild-type *E.coli* using isopycnic sucrose density gradient centrifugation (Figure 4). Appropriate marker enzymes were used to identify the inner membrane (NADH oxidase) and outer membrane (phospholipase A) fractions. PagP activity was clearly localized to the outer membrane.

Expression and in vitro activity of PagP from E.coli

Under our assay conditions, the PagP specific activity of wild-type *Salmonella* membranes using disaccharide acceptors as substrates at 10 μM (150 pmol/min/mg) is ~5- to 10-fold greater than that observed with wild-type *E.coli* membranes (15–30 pmol/min/mg). Therefore, the *pagP* homolog of *E.coli* (*crcA*) (Hu *et al*., 1996) was cloned with its endogenous ribosome-binding site placed behind an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac*-promoter in the expression vector pMS119HE (Strack *et al*., 1992) to create plasmid pCrcHD. Outer membranes were prepared from induced cells transformed with the vector or with pCrcHD, and assayed for palmitate transfer to lipid X, lipid IV_A or Kdo_2–lipid IV_A as acyl acceptors. Expression of *crcA* yielded ~500-fold overproduction of PagP above the background activity. The enzyme utilizes the disaccharides lipid IV_A and Kdo_2–lipid IV_A nearly 3-fold more rapidly under standard assay conditions than the monosaccharide lipid X (Figure 5). Unlike LpxL and LpxM (Brozek and Raetz, 1990), PagP clearly does not depend upon the presence of the two Kdo sugars.

Lipid A is conjugated to the core oligosaccharide and the O-antigen polymer in the inner membrane before it is targeted to the outer membrane (Raetz, 1996). Therefore, an outer membrane enzyme involved in lipid A biosynthesis must utilize fully assembled LPS as a substrate. We examined whether PagP in outer membranes could acylate Kdo_2–lipid A (Re endotoxin), which is the simplest chemotype of LPS capable of supporting the growth of *E.coli* K-12. Kdo_2–[^{32}P]lipid A (hexa-acylated) was

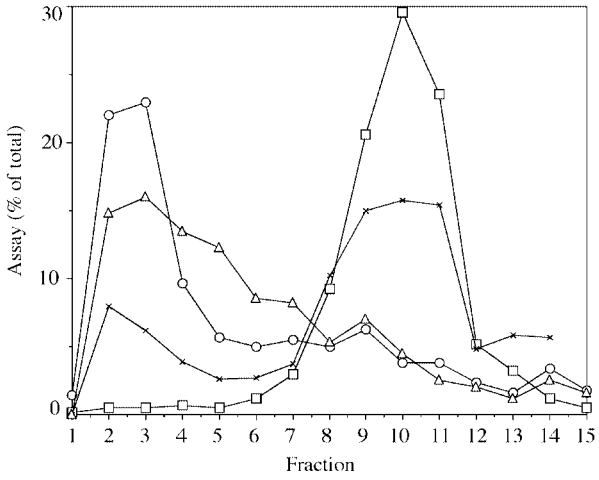

Fig. 4. Outer membrane localization of PagP activity in wild-type *E.coli* membranes. The inner and outer membranes from *E.coli* MC1061 were separated by isopycnic sucrose density gradient centrifugation, and ~0.7 ml fractions were collected. Light scattering material was detected by measuring the A_{550}. The inner and outer membranes were located by measuring NADH oxidase and phospholipase A activities, respectively. PagP activity was measured as described in the legend to Figure 2, but with 12.5 µl portions from each fraction using ^{32}P-labeled lipid IV_A (200 c.p.m./µl) at 100 µM for 220 min. Measurements are expressed as a percentage of the total activity units across the entire gradient. NADH oxidase (squares), phospholipase A (triangles), light scattering (crosses) and PagP (circles).

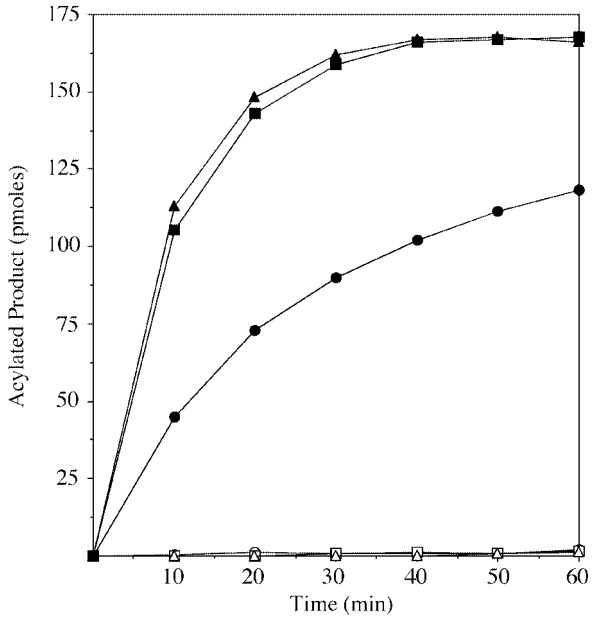

Fig. 5. Relative rates of product formation by *E.coli* PagP-overproducing outer membranes with lipid X, lipid IV_A or Kdo_2–lipid IV_A as acyl acceptors. PagP was assayed as described in the legend to Figure 2 using outer membranes at 10 µg/ml. Each substrate was present at 10 µM in 25 µl reaction volumes. Induced outer membranes were prepared from *E.coli* MC1061 transformed with pMS119HE (open symbols) or pCrcHD (solid symbols). Acyl acceptors are lipid X (circles), lipid IV_A (squares) or Kdo_2–lipid IV_A (triangles).

prepared from the heptose-deficient *E.coli* strain WBB06 (Brabetz *et al.*, 1997). Using induced outer membranes from *E.coli* MC1061 transformed with either pMS119HE or pCrcHD, hepta-acylated Kdo_2–[^{32}P]lipid A was rapidly detected only when PagP was overproduced (Figure 6). This result indicates that PagP is capable of transferring palmitate to hexa-acylated lipid A, as would be present as the hydrophobic anchor of fully assembled LPS in the outer membrane.

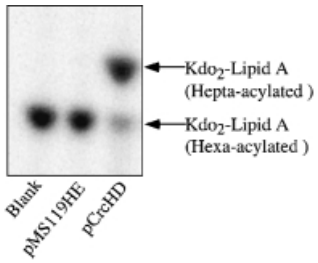

Fig. 6. Kdo_2–lipid A is a substrate for acylation by PagP. Kdo_2–[^{32}P]lipid A (100 c.p.m./µl) at ~10 µM was assayed for PagP activity for 5 min as described in the legend to Figure 2 using induced outer membranes (10 µg/ml) from *E.coli* MC1061 transformed with either pMS119HE or pCrcHD. The TLC plate was developed in the solvent system of chloroform:pyridine:88% formic acid:water (30:70:16:10 v/v) and the separated products were visualized by overnight exposure to a PhosphorImager screen. The Rf values for Kdo_2–lipid A are 0.52 (hexa-acylated) and 0.60 (hepta-acylated). The positions of the two Kdo_2–lipid A derivatives are indicated to the right of the figure.

Expression and activity of His_6-PagP

We have purified the *crcA* gene product in order to establish unequivocally its identity with the palmitoyl transferase enzyme. To achieve massive overproduction of the protein, we used PCR to amplify the *E.coli crcA* gene for cloning behind the T7-RNA polymerase promoter and ribosome-binding site in plasmid pET21a$^+$. Two PCR products were constructed to clone the wild-type *crcA* open reading frame (pETCrcA) and to include a C-terminal His_6 tag (pETCrcAH). Induced membranes from *E.coli* BL21(DE3)/pLysE transformed with pET21a$^+$, pETCrcA or pETCrcAH were prepared and separated by isopycnic sucrose density gradient centrifugation. Whole membranes, inner membranes and outer membranes were analyzed by SDS–PAGE (Figure 7). The majority of both overproduced proteins were recovered in the outer membranes, although a small amount of the overproduced His_6-tagged protein is present in the inner membranes. This appears to result from outer membrane contamination since several porins and OmpA are clearly present in this fraction. The specific activities in outer membranes for both proteins using 10 µM lipid IV_A as the acceptor substrate were identical (3.3 µmol/min/mg) and represent ~10^5-fold overproduction of PagP activity. This finding demonstrates that the C-terminal His_6-tag does not interfere with the expression, outer membrane assembly, or activity of PagP.

In order to establish that the overproduced protein was encoded by *crcA*, we transferred the outer membrane proteins from the gel to a polyvinylidene fluoride membrane for protein micro-sequencing. We focused on the His_6-tagged protein band for this purpose, because it did not migrate with any other proteins in the gel. The first 10 amino acid residues were identified in the order NADEWMTTFR, corresponding to the sequence following a probable 25 amino acid signal peptide at the N-terminus of the *E.coli crcA* gene. Signal peptides similar

to that of *E.coli* PagP are apparent in the homologs currently identified in the genomes of *Y.pestis* (53% identical), *Bordetella* species *pertussis* and *bronchiseptica* (38% identical), and *Salmonella enterica* serovars *typhi*, *paratyphi* and *typhimurium* (75% identical). These can be accessed through DDBJ/EMBL/GenBank and the NCBI microbial genomes database (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html).

Purification and dimeric structure of His_6-PagP

In order to purify the His_6-tagged outer membrane protein by metal chelate affinity chromatography, it was necessary first to solubilize it from the membranes. Two successive solubilization steps with 0.01 and 0.1% lauroyl dimethylamine *N*-oxide (LDAO) in the presence of $MgSO_4$ served to solubilize most cytoplasmic membrane proteins, whereas the PagP activity remained with the insoluble outer membrane proteins. PagP was then solubilized quantitatively (Table I) by increasing the LDAO concentration to 0.5%. The majority of other outer membrane proteins remained insoluble under these conditions. The His_6-tagged protein was observed in the 0.5% LDAO extract by SDS–PAGE and appeared to be >90% homogenous (not shown).

The solubilized His_6-tagged protein was bound to a Ni^{2+}-affinity matrix, washed and then eluted with steps of increasing imidazole concentrations. After the protein peak was pooled and dialyzed, palmitoyl transferase activity was recovered in 68% yield (Table I). The migration of the purified His_6-tagged protein in SDS–PAGE (Figure 8) was found to be heat-modifiable, which is diagnostic of many integral outer membrane proteins (Schnaitman, 1973). The migration in SDS–PAGE of the heat-treated sample corresponds to the predicted molecular weight of the mature His_6-tagged protein (20.0 kDa). The unheated sample migrated further, suggesting that it maintains some secondary structure in the gel. A possible contaminant of roughly twice the molecular weight was also apparent in the unheated sample (Figure 8). We tested whether the purified His_6-tagged protein might actually be a dimer by chemically cross-linking it with glutaraldehyde. Indeed, the glutaraldehyde-treated sample produced a major band that migrated with the apparent contaminant in the untreated His_6-tagged protein. The migration of the heat-modified cross-linked material corresponds exactly to the predicted molecular weight (40.0 kDa) of a dimer of the His_6-tagged protein. The association of the palmitoyl transferase activity with the purified His_6-tagged protein establishes its identity as the *crcA* gene product.

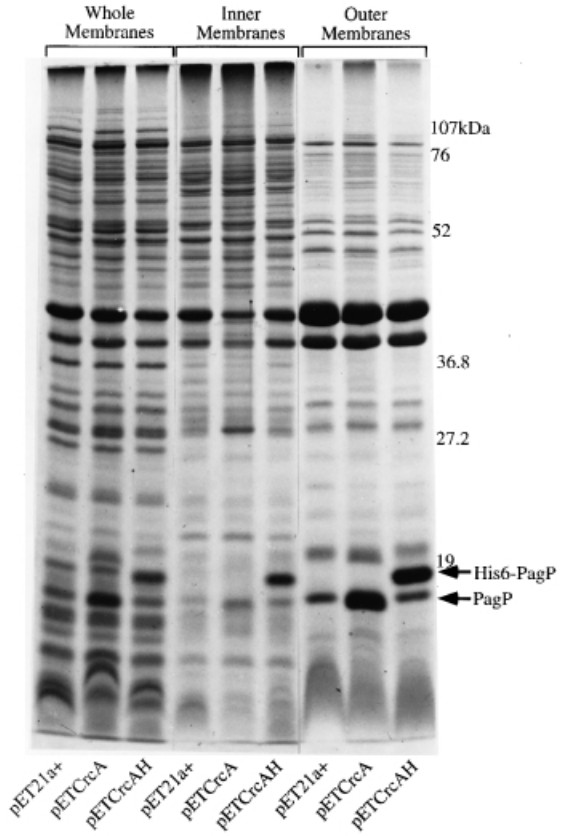

Fig. 7. Outer membrane localization of the PagP and His_6-PagP proteins in induced membranes of *E.coli* BL21(DE3)pLysE transformed with pET21a+, pETCrcA or pETCrcAH. The inner and outer membranes were separated by isopycnic sucrose density gradient centrifugation. Next, 40 μg samples of protein from the whole membranes, the inner membranes, or the outer membranes were solubilized, boiled for 10 min, analyzed by 15% SDS–PAGE and stained with Coomassie Blue dye. The positions of molecular weight standards, PagP and His_6-PagP, are indicated to the right of the gel.

Phospholipid substrate requirements of purified His_6-PagP

Brozek *et al*. (1987) previously established that the *in vitro* acylation of lipid X catalyzed by ethanol-washed *E.coli* membranes exhibited a strong preference for a phospholipid cosubstrate that contained palmitate at the *sn*-1 position, but little preference for the polar head group. In order to confirm that purified His_6-PagP behaved similarly, we examined its specific activity using various phospholipids of defined acyl-chain composition (Table II). Trace activity above background was observed in the absence of added co-substrate, indicating that some phospholipids may remain associated with the purified enzyme. Using a set of phosphatidylcholine (PtdCho) substrates, we observed the expected strong preference for palmitate at the *sn*-1 position. Alterations in acyl-chain length as little

Table I. Solubilization and purification of His_6-PagP

Fraction	Total protein (mg)	Total volume (ml)	Specific activity[a] (μmol/min/mg)	Total units (μmol/min)	% yield
Membranes	34.6	8	2.9	100	100
0.5% LDAO	10.6	8	10.6	113	100+
Ni^{2+}-eluate	0.85	2.5	80.2	68	68

[a]Assayed with 10 μM lipid IV_A and 1 mM *sn*-1-(16:0)-2-(18:1*c*Δ^9)-PtdCho.

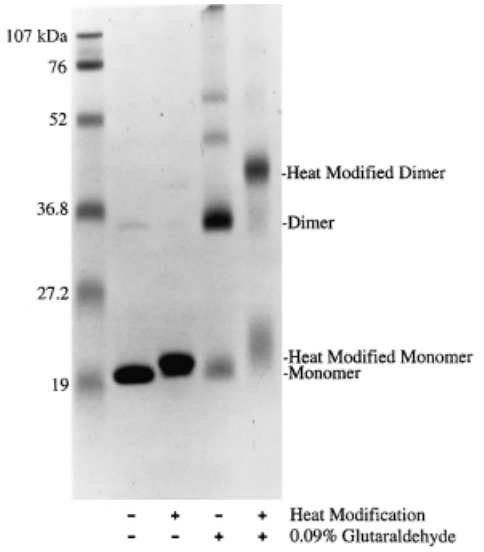

Fig. 8. Heat modification and chemical crosslinking of purified His_6-PagP. Two 17 μg samples of purified His_6-PagP were treated with or without prior incubation in 0.09% glutaraldehyde for 100 min at 25°C. Each sample was then split into two 8.5 μg fractions and solubilized, with or without boiling for 10 min, for analysis by 15% SDS–PAGE. The gel was stained with Coomassie Blue dye. The positions of monomeric and dimeric bands, and their heat-modified derivatives, are indicated to the right of the gel. The positions of molecular weight standards are indicated to the left of the gel.

as one carbon atom reduced the acylation of lipid IV_A by up to 6-fold. The presence of oleate at the *sn*-2 position was beneficial, provided that palmitate was present at the *sn*-1 position. This observation may reflect the unique physical properties imparted by the presence of an unsaturated acyl-chain. Purified His_6-PagP is completely unable to utilize either *sn*-1-(16:0)-2-lyso PtdCho or the *sn*-2/*sn*-3 isomer of dipalmitoyl PtdCho (Table II). Since *E.coli* does not make PtdCho, we examined whether common *E.coli* phospholipids containing palmitate at the *sn*-1 position could be utilized as substrates. Phosphatidylglycerol (PtdGro), phosphatidylethanolamine (PtdEtn), phosphatidylserine (PtdSer) and phosphatidic acid (Ptd-OH) were all effective acyl donors to varying extents (Table II). However, dipalmitoylglycerol (16:0/16:0 Gro) was completely inactive (Table II). We conclude that purified His_6-PagP possesses the same phospholipid substrate specificity as displayed by the enzyme reported by Brozek *et al*. (1987).

Discussion

An important role for lipid A modifications is to provide resistance against CAMPs. A large body of evidence indicates that CAMPs associate with the cell surface of Gram-negative bacteria by electrostatic interactions with negatively charged groups on LPS (Schindler and Osborn, 1979; Rana *et al*., 1991; Bruch *et al*., 1999). Partial neutralization of the negatively charged phosphate groups at the 1 and 4′ positions of lipid A by increased covalent modification with L-4-aminoarabinose and phosphoethanolamine moieties proabably explains the phenomenon of polymyxin B and CAMP resistance in *S.typhimurium* and *E.coli* (Vaara *et al*., 1981; Helander *et al*., 1994). CAMP resistance in Salmonellae depends in part on the PhoP/PhoQ-activated gene *pagP*, which is required for the palmitoylation of lipid A (Guo *et al*., 1998). The transfer of an additional (seventh) acyl-chain to the normally hexa-acylated lipid A may prevent the self-promoted uptake of CAMPs across the hydrophobic bilayer of the outer membrane before they proceed to damage the inner membrane (Guo *et al*., 1998). Our results demonstrate that *pagP* and its homolog in *E.coli* (*crcA*) encode the enzyme (PagP) that is responsible for lipid A palmitoylation.

Table II. Phospholipid requirements of purified His_6-PagP[a]

Phospholipid cosubstrate[b]	Specific activity (μmol/min/mg)
None	1.07
15:0/15:0 PtdCho	5.71
17:0/17:0 PtdCho	3.77
18:1*c*Δ^9/18:1*c*Δ^9 PtdCho	4.14
16:0/18:1*c*Δ^9 PtdCho	29.59
18:1*c*Δ^9/16:0 PtdCho	9.68
16:0/16:0 PtdCho	23.29
16:0/lyso PtdCho	0.51
16:0/16:0 PtdCho (*sn*-2/*sn*-3)	0.73
16:0/16:0 Gro	0.83
16:0/16:0 Ptd-OH	9.82
16:0/16:0 PtdSer	18.20
16:0/16:0 PtdEtn	3.93
16:0/18:1*c*Δ^9 PtdEtn	7.22
16:0/18:1*c*Δ^9 PtdGro	9.18

[a]Assayed with 10 μM lipid IV_A and 1 mM of the specified phospholipid, and using purified His_6-PagP at 10 ng/ml.
[b]Stereochemistry is *sn*-1/*sn*-2 unless indicated otherwise. 15:0, pentadecanoyl; 16:0, palmitoyl; 17:0, heptadecanoyl; 18:1*c*Δ^9, oleoyl.

Escherichia coli PagP is an outer membrane protein that is synthesized with a cleavable type-I signal peptide. We also detected the PagP protein in outer membranes of a *Salmonella* PhoP^c mutant. The outer membrane localization of PagP casts doubt on the physiological significance of its role in the conversion of lipid X to lipid Y (Brozek *et al*., 1987), both of which accumulate in mutants conditionally defective in the gene for the lipid A disaccharide synthase *lpxB*(*pgsB*) (Nishijima and Raetz, 1979; Ray *et al*., 1984). While lipid X is a key cytoplasmic monosaccharide precursor in the lipid A biosynthetic pathway (Raetz, 1996), accumulation of lipid Y in *lpxB* mutants may be secondary to the excessive accumulation of lipid X under non-permissive conditions and/or to outer membrane reorganization under conditions of limited lipid A biosynthesis. Although a regulatory function for palmitoylated precursors of lipid A cannot be ruled out, the most plausible physiological substrate for PagP would appear to be the lipid A moiety of fully assembled LPS in the outer membrane. The simplest chemotype of LPS known to support growth, Kdo_2–lipid A (Re endotoxin), was found to be an acceptor for PagP catalyzed acylation *in vitro*.

The activation of integral outer membrane enzymes in *E.coli* by physical perturbations to the outer membrane is now emerging as a potential mechanism for the maintenance of outer membrane lipid asymmetry. Integral outer membrane enzymes of *E.coli* include the duplicated OmpT and OmpP proteases, which are 87% identical

and hydrolyze peptide bonds between paired basic residues (Sugimura and Nishihara, 1988; Kaufmann *et al*., 1994), as well as the Ca^{2+}-dependent phospholipase OMPLA (Snijder *et al*., 1999; Dekker, 2000). OmpT provides resistance against protamine, a polycationic antimicrobial peptide known to disorganize outer membrane lipids (Stumpe *et al*., 1998). In addition, a *Salmonella* homolog of OmpT is required for resistance to α-helical CAMPs and is regulated in a PhoP/PhoQ-dependent manner (Guina *et al*., 2000). The recently determined crystal structure of the outer membrane phospholipase OMPLA of *E.coli* shows how it hydrolyzes phospholipids in the outer membrane (Snijder *et al*., 1999). Events that disturb the outer membrane can activate OMPLA, which may function to restore outer membrane lipid asymmetry by hydrolyzing phospholipids that migrate into the outer leaflet (Dekker, 2000). PagP is only the third enzyme shown to be an integral outer membrane protein in *E.coli* and is the first example of an outer membrane enzyme of lipid A biosynthesis.

The Gram-negative outer membrane is a major reservoir for Mg^{2+}, which serves to bridge the negative charges between individual LPS molecules (Coughlin *et al*., 1983a,b). The strong Mg^{2+}-dependent lateral interactions between LPS molecules help to maintain outer membrane lipid asymmetry (Nikaido, 1996). Treatment of *E.coli* or Salmonellae with the Mg^{2+} chelating agent ethylenediaminetetraacetic acid (EDTA) disrupts outer membrane lipid asymmetry by selectively stripping 30–50% of LPS molecules from the cell surface, presumably as a result of electrostatic repulsion (Leive *et al*., 1968). Consequently, phospholipids are thought to migrate into the outer leaflet to replace the lost LPS and create patches of phospholipid bilayers within the outer membrane (Nikaido, 1996). Cells treated with EDTA remain viable, but become permeable to lipophilic solutes that cannot normally penetrate the outer membrane (Leive, 1968). OMPLA is dependent on Ca^{2+} for both activity and dimerization, and is unlikely to be fully active at micromolar divalent cation concentrations where large numbers of phospholipid molecules populate the outer leaflet of the outer membrane (Nikaido, 1996). It is conceivable that total hydrolysis of phospholipids under these conditions might be deleterious. PagP activity is independent of divalent cations and may thereby provide a specific response to those perturbations in outer membrane lipid asymmetry induced by Mg^{2+} limitation. The resulting hepta-acylated lipid A should be more firmly anchored to the cell surface, while the lysophospholipid byproduct might be degraded and recycled (Hsu *et al*., 1991). The regulation of *pagP* by the Mg^{2+}-sensing PhoP/PhoQ virulence regulator in pathogenic Salmonellae is consistent with a role for PagP in providing an adaptive response toward Mg^{2+} limitation.

Lipid A is a distinguishing feature of Gram-negative bacteria, and the genomes of ~30 species have revealed key enzymes in the lipid A biosynthetic pathway. However, PagP is narrowly distributed within a subgroup of primarily pathogenic Gram-negative organisms, which include *Y.pestis*, *B.pertussis*, *B.bronchiseptica* and various strains of *Salmonella*. Effective PagP inhibitors may have value in the treatment of infectious diseases by sensitizing pathogenic Gram-negative bacteria to the innate immune response (Guo *et al*., 1998). While such inhibitors would probably not be useful in the treatment of acute infections, they might have value in preventing recurrence. The specificity of PagP for palmitate at the *sn*-1 position of phospholipids provides a rational basis for the design of transition state analog inhibitors. Further investigation of PagP structure and function will be needed for the development of anti-infective agents.

Materials and methods

Materials

$^{32}P_i$ and [γ-^{32}P]ATP were purchased from NEN LifeScience Products Inc. Phospholipids, antibiotics, NADH, 25% glutaraldehyde and Ponceau S were obtained from Sigma. LDAO was obtained from Fluka. Pyridine, methanol and 88% formic acid were obtained from Mallinckrodt. Chloroform and glacial acetic acid were purchased from EM Science. Glass-backed Silica Gel 60 TLC and HPTLC plates (0.25 mm) were from E.Merck, Germany. Bicinchoninic acid protein assay reagents and Triton X-100 were obtained from Pierce. The His-bind resin and buffer kit were obtained from Novagen. QIAprep spin miniprep, Qiaquick PCR purification, and QIAEX II gel extraction kits were obtained from Qiagen. Gibco-BRL custom primers were manufactured by Life Technologies, Inc. *Pfu* polymerase and supercompetent *E.coli* XL1-Blue were obtained from Stratagene. Restriction endonucleases, deoxyribonucleotide triphosphates and dodecylmaltoside were obtained from Boehringer Mannheim.

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table III. Plasmid pKH1 was kindly provided by Dr Nancy J.Trun, National Institutes of Health, Baltimore, MD. Cells were generally grown at 37°C in LB broth, consisting of 10 g of NaCl, 5 g of yeast extract and 10 g of Tryptone per liter. Antibiotics were added when necessary at final concentrations of 12 μg/ml for tetracycline, 10 μg/ml for chloramphenicol, 100 μg/ml for ampicillin, 100 μg/ml for streptomycin and 50 μg/ml for kanamycin.

DNA manipulations

Restriction enzyme digestions, ligations, transformations and DNA electrophoresis were performed according to Sambrook *et al*. (1989). Plasmid pCrcHD was constructed by cloning the 1100 bp *Hin*dIII–*Dra*I fragment carrying *E.coli crcA* from plasmid pKH1 into the IPTG-inducible *tac*-promoter expression vector pMS119HE, which was opened by *Hin*dIII–*Sma*I digestion. PCR gene amplification was performed with 2.5 U of *Pfu* polymerase in a volume of 50 μl of the supplied buffer with 10 ng of pCrcHD as template, 0.2 μM of the appropriate primers (Table IV) and 10 μM dNTPs. After initial denaturation for 7 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 72°C were performed, followed by 5 min at 72°C. The *Nde*I–*Eco*RI-digested PCR product that was amplified from pCrcHD using the primers crcA5Nde and crcA3Eco was cloned into pET21a$^+$ digested with the same enzymes to create plasmid pETCrcA. The C-terminal His_6-tag fusion plasmid pETCrcAH was constructed similarly, except that PCR was performed using the primers crcA5Nde and crcA3Xho, and the product cloned into pET21a$^+$ using *Nde*I–*Xho*I digestion. All cloned PCR products were subjected to double strand DNA sequencing at the Duke University Comprehensive Cancer Center DNA Analysis Facility to confirm the absence of any spurious mutations.

Protein analysis

Protein extracts (40 μg) were solubilized in an equal volume of 2× SDS buffer (Sambrook *et al*., 1989) with or without boiling for 10 min where indicated. SDS–PAGE on 1.5-mm thick 15% acrylamide slab gels was performed with a Bio-Rad Protean II XI apparatus and operated at 90–180 V. Gels were stained with Coomassie Blue dye and destained with 10% acetic acid. The Bio-Rad mini-Protean Transblot apparatus was used to electroblot protein to an Immobilon-P polyvinylidene fluoride membrane (Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid pH 11 with 10% methanol at 100 V (~200–340 mA) for 30 min. The membrane was stained with 0.1% Ponceau S in 1% acetic acid and destained with 1% acetic acid. The band of interest was excised with a razor blade, washed with water and subjected to high sensitivity protein micro-sequence analysis on an ABI model 492A Procise Sequencer at the University of Massachusetts Medical School Worcester Foundation

Table III. Bacterial plasmids and strains used in this work

Plasmid/strain	Description/genotype	Source[a]
Plasmid		
pMS119HE	IPTG-inducible *tac*-promoter expression vector (Ap^R)	Strack *et al*. (1992)
pET21a$^+$	IPTG-inducible T7 RNA polymerase-promoter expression vector (Ap^R)	Novagen
pKH1	pBR322-derived plasmid carrying the 2200 bp *Hin*dIII–*Eco*RI fragment of *E.coli crcAcspEcrcB* locus	Hu *et al*. (1996)
pCrcHD	1100 bp *Hin*dIII–*Dra*I fragment of pKH1 carrying *crcA* cloned into *Hin*dIII–*Sma*I-digested pMS119HE	this study
pETCrcA	600 bp *Nde*I–*Eco*RI PCR product carrying *crcA* cloned into pET21a$^+$	this study
pETCrcAH	600 bp *Nde*I–*Xho*I PCR product carrying *crcA* cloned into pET21a$^+$ with C-terminal His_6 tag	this study
E.coli		
BL21(DE3)pLysE	F^-, *ompT*, *hsdS*$_B$ ($r_B^-m_B^-$), *gal*, *dcm*, λ(DE3), pLysE (Cm^R)	Novagen
XL1-Blue	F^-, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac*, [F′ *proAB*, *lacI*^{q}*ZΔM15*, Tn*10*(Tet^r)][c]	Stratagene
MC1061	F^-, *araD139*, Δ(*ara-leu*)*7697*, Δ(*lac*)*X74*, *galU*, *galK*, *hsdR2* ($r_K^-m_K^+$), *mcrB1*, *rpsL*	Casadaban and Cohen (1980)
MN7	F^-, *rpsL136*, *his-4*, *pgsA444*, *nalA*, *pgsB1(lpxB1)*	Nishijima *et al*. (1981)
WBB06	W3110 *mtl*, Δ(*rfaC-rfaF*)::tet6	Brabetz *et al*. (1997)
S.typhimurium		
ATCC 14028	wild-type *Salmonella enterica* serovar. *typhimurium*	ATCC
CS022	ATCC 14028, *pho-24*(*phoP*^{c}, PhoP constitutive)	Miller and Mekalanos (1990)
LG069	ATCC 14028, *pho-24*, *pagP2*::Tn*10d*-tet, *phoN2*, *zxx6251*::Tn*10d*-cam	Guo *et al*. (1998)

[a]ATCC, American Type Culture Collection.

Table IV. Oligonucleotide primers used in this work

Name	Function	Sequence
T7 promoter	DNA sequencing	5′-TAATACGACTCACTATAGGG-3′
T7 terminator	DNA sequencing	5′-GCTAGTTATTGCTCAGCGG-3′
crcA5Nde	PCR	5′-GGGAATTCCATATGAACGTGAGTAAATATGTC-3′
crcA3Eco	PCR	5′-CCGGAATTCTCAAAACTGAAAGCGCATC-3′
crcA3Xho	PCR	5′-TCCCTCGAGAAACTGAAAGCGCATCCA-3′

Campus Core Laboratory for Protein Microsequencing and Mass Spectrometry. Protein was assayed with the bicinchoninic acid protocol (Smith *et al*., 1985) as described by the manufacturer (Pierce) and using bovine serum albumin as the standard.

Preparation and isolation of lipid substrates

All lipid substrates in aqueous buffer were dispersed prior to use by sonic irradiation for 1 min in a bath sonicator. [^{32}P]lipid X was prepared and isolated from *E.coli* MN7 (Nishijima *et al*., 1981) as described by Radika and Raetz (1988). [4′-^{32}P]lipid IV_A was prepared using [γ-^{32}P]ATP according to the procedure of Garrett *et al*. (1997), and isolated as described by Basu *et al*. (1999). Kdo_2–[4′-^{32}P]lipid IV_A was prepared and isolated according to Basu *et al*. (1999). Laboratory stocks of non-radioactive substrates were prepared and isolated as described for lipid X (Radika and Raetz, 1988), lipid IV_A (Raetz *et al*., 1985) and Kdo_2–lipid IV_A (Brozek *et al*., 1989). Kdo_2–[^{32}P]lipid A (hexa-acylated) was prepared using the heptose-deficient *E.coli* strain WBB06 (Brabetz *et al*., 1997). The cells were grown to an A_{600} of ~1.0 in 100 ml of G56 minimal medium with 0.3 mM potassium phosphate (Ganong *et al*., 1980) containing 5 μCi/ml of $^{32}P_i$. The cells were harvested and lysed by re-suspension in 12 ml of a single phase Bligh/Dyer mixture, containing chloroform:methanol:water (1:2:0.8 v/v), and incubated for 60 min at room temperature. The insoluble material was removed by centrifugation, and the radiolabeled Kdo_2–lipid A found in the supernatant was isolated by chromatography on DEAE cellulose, as described for *E.coli* lipid A (Odegaard *et al*., 1997). Approximately 3 μCi of the $^{32}P_i$ was incorporated into the Kdo_2–lipid A fraction.

Palmitoyl transferase reactions

Palmitoyl transferase assays were performed with varying amounts of membranes or purified His_6-PagP in 25 μl vol. as described by Brozek *et al*. (1987), with minor modifications. The reaction contains 100 mM Tris–HCl pH 8, 10 mM EDTA and 0.25% Triton X-100 with 1 mM *sn*-1-(16:0)-2-(18:1cΔ^9)-PtdCho as acyl donor and 100–200 c.p.m./μl of the ^{32}P-labeled lipid X, lipid IV_A, Kdo_2–lipid IV_A or Kdo_2–lipid A acceptors at 10–100 μM. Reactions were stopped by spotting 5 μl to the origin of a Silica Gel 60 TLC plate. The various chromatographic solvent systems that were used include chloroform:methanol:water:acetic acid (25:15:4:2 v/v), chloroform:pyridine:88% formic acid/water (50:50:16:5 v/v and 30:70:16:10 v/v). Plates were dried and exposed to a Molecular Dynamics PhosphorImager screen overnight to identify and quantify both substrates and products.

Protein expression and membrane isolation

Salmonella membranes were prepared from 25 ml cultures grown to A_{600} = 1–3. *Escherichia coli* membranes were prepared from 100 ml cultures that were grown to mid-logarithmic phase (A_{600} = 0.5–0.6) before addition of 1 mM IPTG, when required, and then grown for an additional 3–4 h. Cells were harvested at 7740 *g* for 10 min at 4°C, washed in 10 ml of ice-cold phosphate buffered saline (PBS; Sambrook *et al*., 1989) and frozen at –80°C. Subsequent steps were performed at 4°C. Cells were thawed in 5 ml of ice-cold PBS and passed twice through a small French Pressure cell at 10 000 psi. Debris was removed by centrifugation at 7740 *g* for 10 min, and membranes were prepared from the supernatant by centrifugation at 318 000 *g* in a Sorval RC-M150GX micro-ultracentrifuge using the S80-AT3 rotor. Membrane pellets were resuspended in 250 μl of PBS by repeated passage through a syringe equipped with a 25 guage needle and stored at –80°C. To separate inner and outer membranes, 200 μl of membranes at 18–28 mg/ml were layered on top of a discontinuous sucrose gradient composed of 0.5 ml of 60%, 1 ml of 55%, 2.4 ml of 50%, 2.5 ml of 45%, 2.4 ml of 40%, 1.4 ml of 35% and 0.8 ml of 30% sucrose in PBS (w/v) and centrifuged for 16 h at

35 000 *g* with a Beckman SW41 rotor. Membrane fractions were assayed directly for the inner membrane NADH oxidase and the outer membrane phospholipase A as described by Zhou *et al*. (1998). Pooled fractions were diluted 2- to 5-fold with water and centrifuged at 318 000 *g* for 20 min to remove sucrose. The inner and outer membrane pellets were resuspended in PBS and stored at –80°C.

Solubilization and purification of PagP

To express the C-terminal His_6-tagged PagP, a single colony of *E.coli* BL21(DE3)pLysE transformed with pETCrcAH was inoculated into 10 ml of medium, grown to an A_{600} = 0.7, and then added directly to 1 l of fresh medium. The culture was then grown to A_{600} = 0.5 before induction with 1 mM IPTG for 4 h. Cells were harvested at 7740 *g* for 10 min at 4°C, washed in 40 ml of ice-cold PBS, and divided into two fractions. The cell pellets were frozen at –80°C. Upon thawing of the two cell pellets in 5 ml each of PBS, a viscous high cell-density suspension was formed due to the presence of the pLysE plasmid. Membranes were then prepared from French Press lysates as described in the preceding section. However, the two membrane fractions were each washed with 5 ml of PBS by resuspending them using syringes equipped serially with 18, 21 and 25 guage needles. Each washed membrane fraction was then resuspended in the same manner using 2 ml of 10 mM Tris–HCl pH 8, 1 mM $MgSO_4$, 0.01% LDAO. The volume of each fraction was adjusted to 4 ml with the same buffer prior to centrifugation at 318 000 *g*. This procedure was repeated twice with the same buffer containing 0.1% LDAO, then 0.5% LDAO, followed by a final resuspension with 10 mM Tris–HCl pH 8 containing 10 mM EDTA and 1.0% LDAO. The supernatant from the 0.5% LDAO solubilization was adjusted to 250 mM NaCl and 5 mM imidazole. It was loaded onto a 1.4 ml bed of charged His-bind resin that had been prepared according to the manufacturer's instructions (Novagen) in a 0.8 × 4 cm open Poly-Prep chromatography column (Bio-Rad), pre-equilibrated with three column volumes of buffer A (10 mM Tris–HCl pH 8, 250 mM NaCl, 0.1% LDAO) containing 5 mM imidazole. After the sample flowed through the column, the resin was washed with 10 column volumes of the pre-equilibration buffer, followed by five column volumes of buffer A containing 25 mM imidazole, and then eluted with serial 1 ml volumes of buffer A containing 50, 75, 100, 125 and 250 mM imidazole. The 75, 100 and 125 mM imidazole fractions were pooled and dialyzed against 1 l of 10 mM Tris–HCl pH 8 containing 0.1% LDAO.

Chemical cross-linking of His_6-PagP was performed by incubating 17 μg of purified protein with 0.09% glutaraldehyde for 100 min at 25°C before the sample was solubilized and split into two fractions for analysis by SDS–PAGE. For most assays, purified His_6-PagP was diluted to an appropriate concentration in 0.1% Triton X-100 prior to its addition to the reaction mixture.

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