Periplasmic Cleavage and Modification of the 1-Phosphate Group of

*Helicobacter pylori* Lipid A

1J. H. Quillen College of Medicine, 2Duke University Medical Center, 3Johns Hopkins University School of Medicine.

An X. Tran1, Mark J. Karbarz2, Xiaoyuan Wang2, Christian R. Raetz2, Sarah C. McGrath3, Robert J. Cotter,3 and M. Stephen Trent1

1Department of Microbiology, Johnson City, Tennessee 37614.

2Department of Biochemistry, Durham, North Carolina 27710.

3Department of Pharmacology and Molecular Sciences, Baltimore, Maryland 21205.

Corresponding author: M. S. Trent at Box 70579, Johnson City, Tennessee 37614.

E-mail: trentms@mail.etsu.edu, Telephone: (423) 439-6293, Fax: (423) 439-8044.

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**Running Title:** Periplasmic addition of phosphoethanolamine to *H. pylori* lipid A
Summary

Pathogenic bacteria modify the lipid A portion of their lipopolysaccharide to help evade the host innate immune response. Modification of the negatively charged phosphate groups of lipid A aids in resistance to cationic antimicrobial peptides targeting the bacterial cell surface. The lipid A of *Helicobacter pylori* contains a phosphoethanolamine (pEtN) unit directly linked to the 1-position of the dissacharide backbone. This is in contrast to the pEtN units found in other pathogenic Gram-negative bacteria, which are attached to the lipid A phosphate group to form a pyrophosphate linkage. This study describes two enzymes involved in the periplasmic modification of the 1-phosphate group of *H. pylori* lipid A. Using an *in vitro* assay system, we demonstrate the presence of lipid A 1-phosphatase activity in membranes of *H. pylori*. In an attempt to identify genes encoding possible lipid A phosphatases, we cloned four putative orthologs of *E. coli* pgpB, the phosphatidylglycerol-phosphate phosphatase, from *H. pylori* 26695. One of these orthologs, Hp0021, is the structural gene for the lipid A 1-phosphatase and is required for removal of the 1-phosphate group from mature lipid A in an *in vitro* assay system. Heterologous expression of Hp0021 in *E. coli* resulted in the highly selective removal of the 1-phosphate group from *E. coli* lipid A, as demonstrated by mass spectrometry. We also identified the structural gene for the *H. pylori* lipid A pEtN transferase (Hp0022). Mass spectrometric analysis of the lipid A isolated from *E. coli* expressing Hp0021 and Hp0022 show the addition of a single pEtN group at the 1-position, confirming that Hp0022 is responsible for the addition of a pEtN unit at the 1-position in *H. pylori* lipid A. In summary, we demonstrate that modification of the 1-phosphate group of *H. pylori* lipid A requires two enzymatic steps.
Introduction

Unlike Gram-positive organisms, Gram-negative bacteria are further guarded from their environment by an asymmetric outer membrane that encapsulates their peptidoglycan. The outer leaflet of the outer membrane is composed mainly of lipopolysaccharide (LPS), the major surface molecule of Gram-negative bacteria. LPS is held in the outer membrane by its hydrophobic anchor lipid A (1,2). Attached to lipid A are the Kdo (3-deoxy-D-manno-octulosonic acid) sugars (Fig. 1) that act as a bridge connecting the lipid A anchor to the core and O-antigen (1-3). The lipid A domain is required to maintain the integrity of the outer membrane barrier (4) and is the bioactive component of LPS that is associated with Gram-negative septic shock (5-7). During Gram-negative bacterial infections, lipid A serves as one of the pathogen-associated molecular patterns (PAMPs) activating the innate immune system (8,9) through the pattern recognition receptor TLR-4 (10-12).

The typical lipid A backbone is a β,1′-6-linked disaccharide of glucosamine that is bis-phosphorylated and multiply acylated. In *E. coli*, the disaccharide backbone is polyacylated with R-3-hydroxymyristate at the 2, 3, 2′, and 3′ positions. The hydroxyl groups of the 2′ and 3′ linked fatty acyl chains are further esterified with laurate (C12) and myristate (C14), respectively (Fig. 1A) (1,2,5). Although a negatively charged hexa-acylated lipid A structure is characteristic in a number of Gram-negative animal pathogens, the lipid A structure can be further modified.

The lipid A of *H. pylori* is quite unusual in that it lacks a 4′-phosphate group, and is tri- or tetra-acylated with either R-3-hydroxystearate (C18) or R-3- hydroxypalmitate (C16) (Fig. 1C) (13-15). Another unusual feature is that the C-1 hydroxyl group of the proximal glucosamine is derivatized with a phosphoethanolamine (pEtN) residue (13-15). This is in contrast with the pEtN units of *E. coli* (16) (Fig. 1), *Salmonella typhimurium* (17), and *Neisseria meningitidis*. 
(18), which are attached to the lipid A phosphate group to form a pyrophosphate linkage. Differences in the *H. pylori* lipid A structure are reflected in its low endotoxicity compared to lipid A produced by Gram-negative bacteria of the *Enterobacteriaceae* family (14,19,20). Pharmacological studies have demonstrated the importance of the phosphate groups and number of fatty acyl chains for cytokine induction via TLR-4 (21,22). In addition to the low potency of *H. pylori* lipid A, the structure of the oligosaccharide portion of its LPS shows structural similarities to Lewis blood group antigens in the gastric mucosa, giving rise to a form of molecular mimicry that helps to camouflage the bacterium (23-25). Therefore, it has been theorized that *H. pylori* has evolved to have a less-inflammatory LPS helping to prolong the chronic infections seen with this organism.

A minor lipid A species found in *H. pylori* is both bis-phosphorylated and hexa-acylated (Fig. 1B) resembling enterobacterial lipid As. We reasoned that the lipid A structure of *H. pylori* is modified following the constitutive lipid A pathway and that these modifications are not regulated given the structure of the major lipid A species shown in Fig. 1A. We report here two proteins, Hp0021 and Hp0022, involved in the variation of the *H. pylori* lipid A structure.

**Experimental Procedures**

**Chemicals and Other Materials** — [γ-32P]ATP and 32P, were obtained from Amersham International. Silica gel 60 (0.25-mm) thin layer plates were purchased from EM Separation Technology (Merck). Yeast extract and tryptone were from Difco. Triton X-100 and bicinchoninic acid were from Pierce. All other chemicals were reagent-grade and were purchased from either Sigma or Mallinckrodt.
Bacterial Strains and Growth Conditions—The bacterial strains and plasmids used in this study are summarized in Table I. *H. pylori* strains J99 and 26695 were obtained from the American Type Culture Collection. Primary plate cultures of *H. pylori* were grown from glycerol stock on blood agar medium at 37 °C for 36-60 h in a microaerobic atmosphere (5% O₂, 10% CO₂, and 85% N₂). The resultant colonies were inoculated in brucella broth (Gibco Diagnostics) supplemented with 10% fetal bovine serum (Hyclone) and vancomycin (10 µg/ml). Cells were grown to an A₆₀₀ of 1.0 at 37 °C under microaerobic conditions for 24-36 h. Prior to every experiment, confirmation of *H. pylori* was performed by both Gram’s stain and urease test.

*E. coli* were typically grown at 37 °C in Luria-Bertani (LB) broth, which consisted of 10 g of NaCl, 10 g of tryptone, and 5 g of yeast extract per liter (26). When required for selection of plasmids, cells were grown in the presence of ampicillin (100 µg/ml) or chloramphenicol (25 µg/ml).

Recombinant DNA Technique—Plasmids were isolated using the Qiagen Spin Prep Kit. Custom primers were obtained from Integrated DNA Technologies. PCR reagents were purchased from Stratagene. PCR clean up was performed using the Qiaquick PCR Purification Kit (Qiagen). DNA fragments were isolated from gels using the Qiaquick Gel Extraction Kit (Qiagen). Restriction endonucleases, T4 DNA ligase, and shrimp alkaline phosphatase were purchased from New England Biolabs. All modifying enzymes were used according to the manufacturers’ instructions.

Cloning of putative *H. pylori* orthologs of *E. coli* pgpB and *hp0022*—*H. pylori* orthologs of *E. coli* pgpB (*hp0021, hp0350, hp0851, and hp1580*) and *Neisseria meningitidis* lptA (60) (*hp0022*) were cloned into pET21a (+) behind the T7lac promoter. The genes were amplified by PCR using *H. pylori* strain 26695 genomic DNA as the template. The forward and reverse primer
sequences of *H. pylori* pgp*B* and *lptA* orthologs are described in Table II. The forward primers contained a clamp region, an *NdeI* site (underlined), and a match to the coding strand starting at the translation initiation start site. The reverse primers were designed with a clamp region, a *BamHI* site (underlined), and a match to the anti-coding strand that included a stop codon. The PCR reaction mixture contained 100 ng of genomic DNA template, 150 ng of each primer, 200 µM of each dNTP, 100 mM Tris-HCl, pH 8.8, 35 mM MgCl₂, 250 mM KCl, and 2.5 units of Pfu DNA polymerase (Stratagene) in a reaction volume of 0.05 ml. The reaction mixtures were subjected to a 90 s denaturation at 95 °C followed by 30 cycles of 95 °C for 60 s, 56 °C for 60 s, 72 °C for 60 s, and a final extension for 10 min at 72 °C, using the Stratagene RoboCyler Gradient 40 PCR system. The PCR products and vector were digested with *NdeI* and *BamHI*, gel-purified, and ligated together. Plasmid constructs derived from pET21a (+) designated pHp0021, pHp0350, pHp0851, pHp1580, and pHp0022 were transformed into XL-1 Blue cells (Stratagene) for propagation of the plasmid. The plasmids were then screened for positive inserts and confirmed by DNA sequencing.

Additionally, *hp0021* was cloned into the expression vector pWSK29 (77). The plasmid pHp0021 was subjected to restriction enzyme digest with *XbaI* and *XhoI*. The resulting fragment containing *hp0021* was ligated into pWSK29 previously digested with *XbaI* and *XhoI* resulting in the vector pWSHp0021.

**High-Copy Expression of Putative PgpB and LptA Orthologs Behind a T7lac Promoter**

*Promoter*—The *H. pylori* PgpB and LptA ortholog constructs derived from pET21a (+) were transformed into NovaBlue(DE3) (Table I) for over-expression of the protein. First, a single colony of *E. coli* harboring either the empty vector control or the recombinant expression plasmid were inoculated into a 50 ml of LB broth and grown overnight (~16 h) in a rotary shaker.
at 37 °C to $A_{600} \sim 1$. The appropriate amount of overnight culture was used to inoculate 200 ml of fresh LB broth with an initial $A_{600}$ of 0.05. Prior to induction with 1mM IPTG, cells were allowed to reach mid-log phase of growth ($A_{600} \sim 0.6$). After induction with IPTG, the cells were left shaking for an additional 4 h. To determine the level of protein expression, membrane proteins were isolated (see below), separated by SDS-polyacrylamide gel electrophoresis, and stained with Coomassie gel code blue staining reagent (Pierce). Unfortunately, expression of Hp0021, Hp0350, Hp0851, Hp1580, or Hp0022 in *E. coli* NovaBlue(DE3) did not result in any distinct protein bands (data not shown).

**Cloning of Putative PgpB Orthologs into a Low-Copy Expression Vector**—PCR amplified pgpB orthologs (*hp0021, hp0350, hp0851, and hp1580*) from *H. pylori* strain 26695 were cloned into pETCoco-2 for low copy replication and protein expression. The forward and reverse primer sequences of pgpB orthologs cloned into pETCoco-2 are described in Table II. The forward primers for pgpB orthologs contained a clamp region, an *Nsi*I site (underlined), and a match to the coding strand beginning at the translation initiation start site. The reverse primers were designed with a clamp region, a *Bst*BI site (underlined), and a match to the anti-coding strand that included a stop codon. Amplification was carried out as mentioned previously. The PCR products and vector were digested with *Nsi*I and *Bst*BI, gel-purified, and ligated together. Plasmid constructs derived from pETCoco-2 designated pHp0021coco, pHp0350coco, pHp0851coco, and pHp1580coco were transformed into NovaF− cells (Novagen) for plasmid propagation. All constructs derived from pETCoco-2 were maintained in the single-copy state with LB broth containing ampicillin (50 $\mu$g/ml) and 0.2% D-glucose at all times to ensure maximum stability, except during culturing for plasmid DNA isolation. Plasmid propagation of pETCoco-2 derived constructs were typically grown by inoculating a single colony of *E. coli*...
containing an empty or recombinant plasmid into 5 ml of LB broth supplemented with ampicillin and 0.2% glucose and grown overnight (~16 h) in a rotary shaker (250 rpm) at 37 °C. The overnight cultures were diluted to 1:50 with fresh LB broth containing ampicillin without glucose since the sugar prevents amplification of the plasmid. Cells were induced with 0.01% L-arabinose for plasmid replication at an A_{600} of 0.4. The cells were allowed to grow for an additional 4 hours prior to harvest for plasmid purification. Plasmid constructs were then screened for positive inserts, confirmed by DNA sequencing, and transformed into *E. coli* K-12 strain HMS174 (DE3) cells (Novagen) for low copy expression.

**Low-Copy Expression of Putative Orthologs of PgpB**—Low copy expression of pETCoco-2 derivatives was performed according to the instructions described by Novagen. Typically, a single colony of *E. coli* harboring either the empty vector control or the recombinant expression plasmid was inoculated into 5 ml of LB broth supplemented with ampicillin and 0.2% glucose, and grown overnight at 37 °C. Overnight cultures were then used to inoculate 20 ml of fresh LB broth containing ampicillin with or without glucose at an initial A_{600} of 0.05. At mid-log phase of growth, the cells were induced with 0.5 mM IPTG and allowed to grow for an additional 3 h before harvesting.

**Isolation and Analysis of Lipid A Species from ^{32}P_i-Labeled Cells**—Cells containing pETCoco-2 derivatives were grown as described above. Upon induction at mid-log phase with IPTG, the cells were labeled uniformly with 2.5 μCi/ml ^{32}P_i for an additional 3 h at 37 °C. Bacteria were collected using a clinical centrifuge and washed with 5 ml of phosphate-buffered saline, pH 7.4. To extract the ^{32}P-labeled phospholipids, the cell pellet was resuspended in 3 ml of a single-phase Bligh/ Dyer mixture (27), consisting of chloroform/methanol/water (1:2:0.8, v/v). After mixing and incubating for 30 min at room temperature, the insoluble residue,
containing the $^{32}$P-labeled lipid A still covalently bound to LPS, was recovered by centrifugation. The residue was subjected to hydrolysis at 100 °C in 12.5 mM sodium acetate buffer, pH 4.5, in the presence of 1% SDS to cleave the Kdo-lipid A linkage (16,28). The $^{32}$P-labeled lipid A species were recovered by two-phase Bligh/Dyer extraction (16,28) and spotted onto a Silica Gel 60 TLC plate (10,000 cpm/lane). The plate was developed in the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). The plate was dried and exposed to a PhosphorImager Screen overnight to visualize the resolved $^{32}$P-lipid A species.

*Cloning and Expression of H. pylori Hp0021 and Hp0022 in a Dual Expression System*—The plasmid constructs, pHp0021 and pETDuet-1, were subjected to restriction enzyme digest with *NcoI* and *BamHI*. The resulting Hp0021 DNA fragment was isolated and ligated into the multiple cloning site (MCS) 1 of pETDuet-1 to derive the plasmid construct pHp0021duet. In addition, the plasmid constructs pHp0022, pETDuet-1, and pHp0021duet were subjected to restriction enzyme digested with *NdeI* and *XhoI*. The resulting Hp0022 DNA fragment was ligated into the MSC2 of both pETDuet-1 and pHp0021duet vectors to generate the plasmid constructs pHp0022duet and pHp0021/Hp0022 respectively. All of the plasmids derived from pETDuet-1 were transformed into XL-1 Blue cells for propagation of the plasmids, screened for positive inserts, and confirmed by DNA sequencing. The plasmids pHp0021duet, pHp0022duet, or pHp0021/Hp0022 were then transformed into the *E. coli* expression strain NovaBlue(DE3) and overexpressed as previously described.

*Cloning and Expression of the E. coli Late Acyltransferases, LpxL and LpxM Behind a T7lac promoter*—The *lpxL* structural gene was obtained by PCR using W3110 genomic DNA as the template. The *lpxM* structural gene was cloned and the protein over-expressed as previously described (29). Primer sequences for *lpxL* are described in Table II. The *lpxL* PCR product was
digested with *Nde*I and *BamHI* and ligated into the similarly digested vector, pET21a (+). The resulting plasmid, pHtrB, was transformed into BLR(DE3)/pLysS cells (Novagen) for protein expression. Cells were grown in LB broth at 37 °C to A<sub>600</sub> of ~ 0.6, followed by induction with 1 mM IPTG and further incubation at 37 °C for an additional 4 h.

*Preparation of Cell-Free Extracts, Double-Spun Cytosol, and Washed Membrane*—Typically, 500 ml of *H. pylori* or 200 ml of *E. coli* cultures were grown to an A<sub>600</sub> of 1.0 at 37 °C and harvested by centrifugation at 6,000 x g for 30 min. All samples were prepared at 4 °C. Cell-free extract, double-spun cytosol, and washed membranes were prepared as previously described (30) and were stored in aliquots at – 20 °C. Protein concentration was determined by the bicinchoninic acid method (31), using bovine serum albumin as the standard.

*Preparation of Radiolabeled Substrate*—The substrate [4′-<sup>32</sup>P]lipid IV<sub>4</sub> was generated from 100 μCi of [γ-<sup>32</sup>P]ATP and the tetraacyl-disaccharide 1-phosphate lipid acceptor, using the over-expressed 4′-kinase present in membranes of *E. coli* BLR(DE3)/pLysS/pJK2 (30,32,33), as previously described (30,33). Kdo<sub>2</sub>[4′-<sup>32</sup>P]lipid IV<sub>4</sub> was prepared by adding purified *E. coli* Kdo transferase (WaaA) immediately after the 4′-kinase, as previously described (33,34). To prepare Kdo<sub>2</sub>[4′-<sup>32</sup>P]lipid A (dilauroyl-Kdo<sub>2</sub>[4′-<sup>32</sup>P]lipid IV<sub>4</sub>), lauroyl-ACP and *E. coli* overexpressed LpxL and LpxM (29) membranes were used in tandem with the Kdo transferase reaction under the following conditions: 50 mM Hepes pH 7.5, 0.1% Triton X-100, 50 mM NaCl, 50 mM MgCl<sub>2</sub>, 0.1 mg/ml BSA, 13.3 μM Lauroyl-ACP, and 0.05 mg/ml of BLR(DE3)/pLysS/pMsbB and BLR(DE3)/pLysS/pHtrB membranes. Once the reaction was complete the substrate was isolated as previously described (33).

*Assay of the 1-phosphatase*—The 1-phosphatase activity from Hp0021 was assayed under optimized conditions in a 10-μl reaction mixture containing 50 mM MES, pH 6.0, 0.2% Triton
X-100, and either 5 μM [4′-32P]lipid IV_A, Kdo_2-[4′-32P]lipid IV_A, or Kdo_2-[4′-32P]lipid A (each at ~3000-5000 cpm/nmol) as the substrate. Washed membranes (0.005-1.0 mg/ml) were employed as the enzyme source, as indicated. Dephosphorylation reactions were incubated at 30 °C for the indicated times. Enzymatic reactions were terminated by spotting 4.5 μl portions of the mixtures onto silica gel 60 TLC plates, and the plate dried under a cool air stream for 20 min.

When [4′-32P]lipid IV_A was employed as the substrate, reaction products were separated using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v). For reactions containing either Kdo_2-[4′-32P]lipid IV_A, or Kdo_2-[4′-32P]lipid A as the substrate, the TLC plates were developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10, v/v). After drying and overnight exposure of the plate to a PhosphorImager Screen, the product formation was detected and analyzed using a Bio-Rad Molecular Imager PhosphorImager equipped with Quantity One Software. The enzyme activity was calculated by determining the percentage of the substrate converted to product.

**Separation of Inner and Outer Membranes**—Membranes isolated from *E. coli* strain NovaBlue(DE3)/pHp0021 were separated by isopycnic sucrose gradient centrifugation. First, washed membranes were prepared as described above and were resuspended in 10 mM Hepes, pH 7.0, containing 0.05 mM EDTA at a protein concentration of 1-3 mg/ml. Membranes (2.2 ml) were layered on to a seven-step gradient, as previously described (35,36), and subjected to ultracentrifugation at 35,000 rpm in a Beckman SW41 rotor for 19 h at 4 °C. The gradient was collected in ~ 0.5 ml fractions. Each fraction was then assayed for NADH oxidase, as the inner membrane marker, and for phospholipase A, as the outer membrane marker, as previously described (28). The amount of protein in each fraction was determined using the bicinchoninic
acid assay (31). Each fraction was also assayed for 1-phosphatase activity using the standard conditions described above.

**Large Scale Isolation of Lipid A from E. coli Cells Expressing H. pylori Hp0021 and Hp0022**—Cultures of *E. coli* strain NovaBlue(DE3) were grown in 1 liter of LB medium at 37 °C containing either pDuet-1, pHp0021duet, pHp0022duet, or pHp0021/Hp0022. The cultures were allowed to reach $A_{600} \sim 0.6$ prior to induction with 1 mM IPTG and further incubated for an additional 4 h at 37 °C. Cells were harvested by centrifugation at 6000 x g for 15 min and washed once with phosphate-buffered saline (37). The final cell pellets were resuspended in 40 ml of phosphate-buffered saline. Lipid A was released from cells and purified as previously described (16,38) and stored frozen at −20 °C.

**Purification of the 1-phosphatase Reaction Product Generated In Vitro from Kdo$_2$-Lipid**

A—A large-scale 1-phosphatase reaction mixture (20 ml) was prepared by assembling the following components: 50 mM MES, pH 6.0, 0.25% Triton X-100, 50 µM of Kdo$_2$-lipid A (39), and 0.3 mg/ml of membrane proteins from NovaBlue(DE3)/pHp0021(39). The reaction was initiated by the addition of the enzyme and incubated at 30 °C for 3 h. The reaction mixture was then converted into a two-phase Bligh-Dyer consisting of chloroform/methanol/0.05 M HCl (2:2:1.8 v/v). The phases were separated by centrifugation at 4000 x g for 10 min. The lower phase was removed, and the resulting upper phase was extracted a second time by the addition of lower phase from a fresh two-phase acidic Bligh-Dyer system. The lower phases were pooled and pyridine (1 drop/4 ml) was added to neutralize the minor amount of HCl carried over during the extraction process. The sample was dried by rotary evaporation, dissolved in 5 ml of chloroform/methanol/water (2:3:1 v/v/v), and applied to a 1 ml DE52 cellulose column equilibrated with the same solvent mixture. Following sample loading, the column was washed
with 5 column volumes of chloroform/methanol/water (2:3:1 v/v). The bound phospholipids and lipid A were eluted with 1 ml steps of chloroform/methanol/ammonium acetate (2:3:1 v/v), with increasing ammonium acetate concentrations of 60, 120, 240, and 480 mM successively in the aqueous component. For each step elution, 1 ml fractions were collected. The presence of lipids was determined by spotting 10 µl of each fraction onto a TLC plate followed by developing in chloroform, pyridine, 88% formic acid, water (30:70:16:10 v/v). After drying, lipids were visualized by spraying the plate with 10% sulfuric acid in ethanol and charring. All fractions containing 1-dephosphorylated-Kdo2-lipid A were pooled and converted to a two-phase Bligh/Dyer mixture by addition of appropriate amounts of chloroform and water. The lower phase was dried under a stream of N2 and stored at –20°C.

**Mass Spectrometry of Lipid A Species**—Mass spectra of the purified lipids were acquired in the negative and positive linear mode using a matrix-assisted laser desorption-ionization/time of flight (MALDI/TOF) mass spectrometer (AXIMA-CFR, Kratos Analytical, Manchester, UK), equipped with a nitrogen laser (337 nm). The instrument was operated using 20-kV extraction voltage and time-delayed extraction, providing a mass resolution of about ± 1 atomic mass units for compounds with Mr ~ 2000. Each spectrum represented the average of 100 laser shots. Saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) served as the matrix in negative and positive modes. The samples were dissolved in chloroform/methanol (4:1, v/v) and deposited on the sample plate followed by an equal portion of matrix solution (0.3 µl). The sample was dried at 25°C prior to mass analysis.
Results

1-phosphatase Activity in Membranes of Wild-Type Virulent Strains of H. pylori

Membranes from virulent strains of H. pylori were isolated and assayed with [4′-32P]lipid IVₐ, a key lipid A precursor substrate, for 1-phosphatase activity. As shown in Fig. 2, membranes from both H. pylori strains 26695 and J99 display 1-phosphatase activity, denoted as 1-Dephospho-[4′-32P]lipid IVₐ (Fig. 2, lane 3, 4, 6, and 7). Removal of the 1-phosphate group from [4′-32P]lipid IVₐ increases the hydrophobicity of the lipid substrate, resulting in a faster migrating lipid species when analyzed by thin layer chromatography (TLC) in the solvent chloroform, pyridine, 88% formic acid, and water (50:50:16:5 v/v). The phosphatase activity seen in Fig. 2 is not a result of 4′-phosphatase activity, because 4′-phosphatase activity would catalyze the release of inorganic 32P from the 4′-position of lipid A when analyzed by TLC. However, a slower migrating species, lipid IV₉, was detected from membranes of wild-type E. coli strain W3110 after 3 h at 30 °C (Fig. 2, lane 5). Lipid IV₉ is generated by the addition of a palmitate chain to the amide linked β-hydroxmyristate residue at position 2 of lipid IVₐ (40,41) that is catalyzed by the E. coli outer membrane protein, PagP (40,42-44). The lipid A of E. coli normally contains phosphate groups at both the 1 and 4′ positions (5) and is not dephosphorylated prior to the addition of a pEtN or L-Ara4N. As a result, membranes from wild-type E. coli strain W3110 do not show 1-phosphatase activity (Fig. 2, lane 2 and 5).

Identification of the H. pylori 1-Phosphatase (Hp0021) from Putative Orthologs of E. coli PgpB

Recently, Karbarz et al. (2003) identified LpxE, a phosphatase that selectively removes the 1-phosphate group of R. leguminosarum lipid A. Discovery of LpxE revealed that it is distantly related to the membrane-bound phosphatidylglycerol phosphatase in E. coli (PgpB) (45-48). Therefore, we searched the COG protein database (49-52) using E. coli PgpB to identify

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possible *H. pylori* lipid phosphatases. COG0671 contains four putative orthologs of PgpB in *H. pylori* strain 26695, designated Hp0021, Hp0350, Hp0851, and Hp1580. The *H. pylori* PgpB orthologs were examined for 1-phosphatase activity by expressing the putative lipid phosphatases in the *E. coli* K-12 strain NovaBlue (DE3). Membranes isolated from NovaBlue (DE3) containing either pET21a (+) (vector control), pHp0021, pHp1580, pHp0851, or pHp0350 were assayed with [4′-32P]lipid IVα for 1-phosphatase activity (Fig. 3). NovaBlue (DE3) membranes expressing Hp0021 displayed a considerable amount of 1-phosphatase activity (Fig. 3, lane 3) as compared with membranes from cells containing the vector control (Fig. 3, lane 2). In contrast, NovaBlue (DE3) membranes expressing either Hp0350 or Hp1580 (Fig. 3, lane 4 and 5) did not demonstrate any 1-phosphatase activity. NovaBlue (DE3) membranes expressing Hp0851 (Fig. 3, lane 6) did display minor amounts of 1-phosphatase activity; however, further examination revealed that the activity was non-specific (see below).

**Assay Conditions and Catalytic Properties of Hp0021**—The Hp0021 protein is predicted to be 190 amino acids long and contains three membrane-spanning regions (see http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). The 1-phosphatase activity of Hp0021 is absolutely dependent on the presence of the nonionic detergent, Triton X-100, with optimal activity at 0.2% detergent. The pH optimum is 6.0, but significant activity is observed from pH 5.5 to 8.0. Divalent cations are not required, and EDTA has no effect on activity. The substrate specificity of Hp0021 is relatively broad (Fig. 4A) and does not require the Kdo moiety or complete acylation for activity, as indicated by the higher amount of activity with [4′-32P]lipid IVα than with either Kdo2-[4′-32P]lipid IVα or Kdo2-[4′-32P]lipid A. Formation of the 1-dephosphorylated reaction products is linear with both protein concentration and time. As shown in Fig. 4B, the 1-dephosphorylation of 5 μM [4′-32P]lipid IVα by 0.01 mg/ml
NovaBlue(DE3)/pHp0021 membranes is linear with time for approximately 40 min at 30 °C. Secondly, the complete 1-dephosphorylation of 5 µM [4′-32P]lipid IVₐ is seen after 1.5 h when using 0.05 mg/ml of membrane protein (Fig. 4A). Overall, the *H. pylori* lipid A 1-phosphatase is a very stable enzyme having a specific activity of ~ 170 nmol/min/mg with lipid IVₐ as the substrate and *E.coli* membranes over-expressing Hp0021 as the enzyme source.

**Low-Copy Expression of H. pylori PgpB Orthologs in E. coli**—Assay of *E. coli* membranes expressing Hp0851 showed minor amounts of 1-phosphatase activity (Fig. 3, lane 6). To confirm that Hp0021 functions as a lipid A phosphatase and to ensure that Hp0851 phosphatase activity was non-specific, we isolated the lipid A from *E. coli* expressing the individual supposed *H. pylori* PgpB orthologs. The putative lipid phosphatases were cloned into the low-copy expression vector pETCoco-2 to generate the following plasmids: pHp0021coco, pHp1580coco, pHp0851coco, and pHp0350coco. A low-copy expression system was utilized to avoid non-specific phosphatase activity generated from high levels of protein expression. Cultures of the *E. coli* K-12 strain HMS174 (DE3) containing one of the pETCoco-2 derived plasmids were labeled with 32P_i and the lipid A fraction isolated to determine possible modifications in vivo.

HMS174 (DE3) containing the pETcoco-2 vector control produced the typical 32P-labeled lipid A species seen in wild-type *E. coli* K-12, 1,4′-bis-phosphate lipid A and 1-pyrophosphate lipid A. The latter contains a pyrophosphate at the 1-position of the disaccharide (Fig. 1) and represents approximately one-third of the lipid A found in *E. coli* grown on nutrient broth (16,28). Low-copy expression of Hp0021 resulted in the production of only 1-dephosphorylated lipid A (Fig. 5, lane 2) illustrating that Hp0021 functions as a lipid A phosphatase. 32P-labeled lipid A fractions isolated from cells containing pHp0350coco, pHp0851coco, and pHp1580coco did not contain any significant amounts of 1-dephosphorylated lipid A species (Fig. 5 lanes 3, 4,
and 5). The minor amount of 1-dephosphorylated lipid seen in lanes 3-5 arises as a decomposition product during mild acid hydrolysis at 100 °C used to free lipid A from LPS (16, 17, 36). An increase in Hp0851 expression by increasing copy number of the pHp0851coco plasmid did not result in any production of dephosphorylated lipid A species (data not shown), confirming that only Hp0021 functions as a lipid A 1-phosphatase in vivo. Bacteria showed normal growth during expression of either Hp0021 or Hp1580. However induction of Hp0851 and Hp0350 resulted in a decrease in growth rate (data not shown). Although we were unable to isolate as much 32P-labeled lipid A from cells expressing Hp0851, it appeared that HMS174(DE3) containing pHp0851coco did not produce the 1-pyrophosphorylated lipid A species.

MALDI/TOF Mass Spectrometry of the 1-Phosphatase Reaction Product—To confirm that hp0021 is the structural gene for H. pylori lipid A 1-phosphatase, the reaction product utilizing mature Kdo2-lipid A as the substrate was analyzed by MALDI-TOF mass spectrometry in the negative mode. MALDI-TOF mass spectrometry of the initial substrate E. coli Kdo2-lipid A revealed a major ion peak at \( m/z \) 2238.7 atomic mass units with additional minor ions at \( m/z \) 2019.3 and 1798.7 atomic mass units (Fig. 6, panel A). The signal at \( m/z \) 2238.7 atomic mass units is interpreted as \([M – H]^-\) of the predominant hexa-acylated Kdo2-lipid A 1,4′-bis-phosphate molecule (Fig. 6A), characteristic of E. coli K-12. The peaks at \( m/z \) 2019.3 and 1798.7 atomic mass units are interpreted as \([M – H–Kdo]^-\) and \([M – H–Kdo2]^-\) respectively, and correspond to the loss of the labile Kdo moiety during mass spectrometry (Fig. 6A). MALDI/TOF mass spectrometry of the Hp0021 reaction product revealed a predominate peak at \( m/z \) 2158.4 atomic mass units confirming the enzymatic removal of the 1-phosphate group (Fig. 6B) from the Kdo2-
lipid A substrate. Again, the minor peaks at \( m/z \) 1938.9 and 1718.5 arise from the loss of either one or two Kdo moieties from the 1-dephosphorylated reaction product respectively (Fig. 6B).

**Identification of the Structural Gene Encoding the H. pylori Lipid A pEtN Transferase (Hp0022)**—We have demonstrated that Hp0021 is responsible for removing the 1-phosphate group from the proximal glucosamine of lipid A. However, the major lipid species of *H. pylori* has a pEtN unit directly linked to the 1-position of the disaccharide backbone (Fig. 1C). This suggests that the modification of *H. pylori* lipid A at the 1-position also requires a lipid A pEtN transferase. Recently, Cox et al. identified NMB1638, renamed LptA, as a pEtN transferase specific for *Neisseria meningitidis* lipid A based upon loss of pEtN from lipid A in an *lptA* mutant (53). Also, it has been shown *in vitro* that *E. coli* YjbB (EptA) transfers pEtN to *E. coli* lipid A precursors (54). Examination of the COG database using either *N. meningitidis* NMB1638 or *E. coli* YjbB to search for orthologs in *H. pylori* strain 26695 revealed two possible orthologs designated Hp0022 and Hp1417m.

We decided to investigate Hp0022 as the possible *H. pylori* lipid A pEtN transferase primarily because of its homology to LptA (E value < 10\(^{-48}\) using BLASTp) and also, because its structural gene is located in a putative operon with *hp0021* in the *H. pylori* genome. Hp0022 is estimated to be 521 amino acids long and is predicted to contain multiple membrane-spanning regions (see [http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html](http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html)). Membranes isolated from NovaBlue (DE3) containing either pET21a (+) or pHp0022 were assayed for pEtN transferase activity using the substrates \([4'-32P]\)lipid IV\(_A\), Kdo\(_2\)\[-\([4'-32P]\)lipid IV\(_A\), and Kdo\(_2\)\[-\([4'-32P]\)lipid A. Assaying Hp0022 with lipid A precursor substrates that were still phosphorylated at the 1-position did not demonstrate any 1-pEtN transferase activity when analyzed by TLC (data not shown). This data
suggest that the activity of Hp0022 may require a lipid A substrate that is initially
dephosphorylated at the 1-position prior to the addition of a pEtN.

To demonstrate that the 1-phosphate group modification of \textit{H. pylori} may require two
enzymatic steps, we developed a dual protein expression system. The target proteins were
expressed under the control of two separate T7\textit{lac} promoters by cloning \textit{hp0021} and \textit{hp0022} into
two separate multiple cloning sites of pETDuet-1 giving the plasmid pHp0021/Hp0022. In
addition, Hp0021 and Hp0022 were individually cloned into pETDuet-1 giving the plasmids
pHp0021duet and pHp0022duet. Separation of 32P-labeled lipid A species from NovaBlue (DE3)
containing the vector pHp0021duet clearly demonstrates again that Hp0021 is responsible for the
presence of 1-phosphatase modified lipid A species (Fig. 7, lane 2). However, cells containing
the pDuet-1 vector control contained the typical 1, 4′-bisphosphorylated and the 1-
pyrophosphorylated \textit{E. coli} lipid A species. As demonstrated in Fig. 7, lane 3, 32P-labeled lipid
A species isolated from NovaBlue (DE3) containing pHp0022duet were similar to those found in
cells containing the pDuet-1 vector control suggesting that Hp0022 alone does not modify \textit{E. coli}
lipid A. 32P-labeled lipid A species isolated from NovaBlue (DE3) containing the dual
expression construct, pHp0021/Hp0022, revealed three lipid species; 1, 4′-bis-phosphorylated
lipid A, 1-dephosphorylated lipid A, and lipid A containing a pEtN group at the C-1 position (see
Fig. 8). Removal of the 1-phosphate group from 1, 4′-\textit{bis}-phosphorylated lipid A followed by
the addition of 1-pEtN unit generated a lipid species that migrates in a similar fashion to 1-
pyrophosphate lipid A (Fig. 7, lane 4). Therefore, mass spectrometry was employed to
differentiate between the two lipid species and confirm that \textit{hp0022} encodes for a pEtN
transferase acting after 1-dephosphorylation of \textit{H. pylori} lipid A.
The lipid A of *E. coli* strain NovaBlue (DE3) containing either pETDuet-1, pHp0021duet, pHp0021duet, or pHp0021/Hp0022 was isolated and analyzed by MALDI/TOF mass spectrometry. The lipid A of NovaBlue (DE3) containing the control vector, pETDuet-1, consisted primarily of the hexa-acylated bis-phosphate species with MALDI/TOF analysis resulting in a major peak at \( m/z \) 1798.4 (16,38) (Fig. 8A). Mass spectrometry analysis of NovaBlue (DE3) containing the control vector also revealed the presence of hexa-acylated pyrophosphate (data not shown). Similar results were seen upon examination of the lipid A fraction of NovaBlue (DE3) expressing Hp0022 (Fig. 8C). As expected, expression of Hp0021 resulted in the loss of a single phosphate unit from lipid A (Fig. 8B), as indicated by \([M-H]^-\) at \( m/z \) 1719.0 in the negative mode. Furthermore, the positive ion spectrum showed the presence of \( B_1^+ \) ion at \( m/z \) 1087.3 indicating that the distal portion of the lipid A was not dephosphorylated (Fig. 8E). Mass spectrometry analysis of lipid A isolated from cells over-expressing both Hp0021 and Hp0022 demonstrated two ion peaks at \( m/z \) 1719.0 and 1840.9 atomic mass units in the negative mode (Fig. 8D). The ion peak at \( m/z \) 1719.0 corresponds to the loss of a phosphate group of lipid A. However, the ion peak at \( m/z \) 1840.9 correlates to the addition of a pEtN group that is interpreted as the addition of a pEtN unit to previously dephosphorylated lipid A. Therefore, based on these data, it can be concluded that the modification of the 1-phosphate group of *H. pylori* lipid A requires two enzymatic steps: (1) removal of the 1-phosphate group by a specific phosphatase (Hp0021) and (2) addition of a pEtN unit directly to the glucosamine disaccharide backbone by a pEtN transferase (Hp0022).

**Lipid A Transport Across the Inner Membrane is Required for Hp0021 Catalyzed 1-Dephosphorylation**—Kdo \(_2\)-lipid A is synthesized on the cytoplasmic face of the inner membrane followed by the addition of the core oligosaccharide. The core-Kdo \(_2\)-lipid A molecule is then
transported across the inner membrane by the essential flippase, MsbA. As previously described by Doerrler and co-workers, MsbA-dependent transport of lipid A across the inner membrane is lost in the *E. coli* temperature-sensitive mutant WD2 by shifting cells from 30 to 44°C for 30 min during mid log phase of growth. We have confirmed that Hp0021 1-phosphatase activity is localized to the inner membrane fraction based upon isopycnic density gradient separation of membranes from NovaBlue (DE3) expressing Hp0021 (data not shown). Using WD2, we investigated whether or not the active site of Hp0021 was facing the periplasmic or cytosolic face of the inner membrane.

*E. coli* WD2 and its parent strain W3110A were transformed with either pWSK29 or pWSHp0021 (pWSK29 expressing Hp0021). Cells were grown at the permissive temperature of 30°C until A₆₀₀ of 0.6-0.7 following a temperature shift to 44°C for 30 min to inactivate MsbA-dependent lipid A transport. Cells were then labeled with 4 µCi/ml ³²Pᵢ for 20 min and the newly synthesized ³²P-labeled lipid A species isolated and separated by TLC. At 30°C, W3110A (non-temperature sensitive control) and WD2 synthesized solely 1-dephosphorylated lipid A species (Fig. 9) when containing the plasmid pWSHp0021. Shifting W3110A expressing Hp0021 to 44°C had no effect on production of 1-dephosphorylated lipid A. However, inactivation of MsbA at 44°C in WD2/pWSHp0021 resulted in the loss of 1-dephosphorylation (Fig. 9) indicating that the *H. pylori* 1-phosphatase active site is oriented towards the periplasm. Recently, Wang et al. showed a similar result using a lipid A 1-phosphatase cloned from *Franciscella novicida* (55).
Discussion

The LPS of *H. pylori* is distinct from enterobacterial LPS in having a unique lipid A structure and many types of O-antigen polysaccharide, including Lewis antigen structures (23-25,56,57). *H. pylori* lipid A shows very low endotoxic properties (14,19,20) and has longer fatty acyl chains and fewer phosphate residues (Fig. 1C) (13,14,19). A number of Gram-negative bacteria modify the phosphate groups of their lipid A (58). Recently, Lee and co-workers has shown that addition of pEtN to the 1-phosphate of *Salmonella enterica* is important for resistance to the antimicrobial peptide polymyxin (72). In the case of *H. pylori*, the C-1 hydroxyl group of the proximal glucosamine sugar is derivatized with pEtN (13,14,19) (Fig. 1C). We report here that the pEtN moiety found at the C-1 position of *H. pylori* lipid A arises from the removal of the lipid A 1-phosphate group followed by the addition of pEtN to form a glycosidic phosphodiester linkage.

Recently, Karbarz and co-workers reported the cloning of a membrane bound enzyme, LpxE, which removes the 1-phosphate group of *R. leguminosarum* lipid A (46). In *R. leguminosarum*, the removal of the 1-phosphate group is followed by oxidation of the proximal sugar by the outer membrane enzyme LpxQ (59,60). LpxE shares sequence homology to *E. coli* PgpB (45,47,48), primarily in the conserved active site motif (61) found in many lipid phosphatases. Examination of the COG database shows that *H. pylori* strain 26695 contains four putative PgpB orthologs designated as Hp0021, Hp0350, Hp0851, and Hp1580. We had already detected the presence of a 1-phosphatase activity in membranes of *H. pylori* (Fig. 2). However, by assaying membranes of *E. coli* expressing the individual *H. pylori* PgpB orthologs using key $^{32}$P-labeled lipid A precursors as substrates, we showed that Hp0021 functions as a lipid A 1-phosphatase (Fig. 3).
Both LpxE and Hp0021 have multi-transmembrane domains and, like other lipid phosphatases require detergent for activity (45,61,62). Hp0021 was capable of removing the 1-phosphate group from various lipid A precursors, including mature Kdo2-lipid A (Figs. 4 and 6). Membranes from wild type *H. pylori* strains 26695 and J99 catalyzed the 1-dephosphorylation of lipid IV₅ at a specific activity of ~ 1.4 nmol/min/mg whereas *Rhizobium* membranes showed a specific activity of only 0.014 nmol/min/mg. In comparison, membranes of *E. coli* over-expressing Hp0021 showed a specific activity of 170 nmol/min/mg resulting in more than a 100-fold increase over wild type levels. Interestingly, expression of Hp0021 in *E. coli* K-12 strains NovaBlue(DE3) (Fig. 7 and Fig. 8B) and HMS174(DE3) (Fig. 5) resulted in nearly 100% production of 1-dephosphorylated lipid A with no significant effects on bacterial growth, suggesting that removal of the 1-phosphate group of lipid A is not toxic to growth under normal laboratory conditions. The ability to generate *E. coli* strains lacking the 1-phosphate group of lipid A may prove to be useful for the development of specific vaccines. Monophosphorylated lipid A has been shown to be an effective adjuvant retaining the immunostimulatory properties of LPS but with greatly reduced toxic effects (63-65).

We also have presented data herein suggesting that Hp0022 functions as the *H. pylori* lipid A pEtN transferase. However, enzymatic data using defined substrates will be needed to confirm the function of Hp0022. The *hp0021* and *hp0022* genes appear to form a typical prokaryotic operon since the individual open reading frames are separated by only 10 bp, and the genes are unidirectionally transcribed beginning with *hp0022*. Hp0022 shares homology with the recently described LptA that is predicted to serve as a putative lipid A pEtN transferase in *N. meningitidis* (53). Based upon topology prediction programs (see http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), Hp0022 has six transmembrane regions.
with the soluble C-terminal region predicted to be located in the periplasmic region of the cell envelope. Expression of Hp0022 alone in an *E. coli* K-12 strain did not result in modification to the bis-phosphorylated *E. coli* lipid A structure. However, expression of both Hp0021 and Hp0022 together using a dual expression system resulted in the production of a lipid A species modified at the C-1 position with pEtN (Figs. 7 & 8). These findings confirmed our hypothesis that *H. pylori* 1-phosphate group modification is a two-step process. One would expect a similar mechanism to occur in the Gram-negative pathogen *Porphyromonas gingivalis*, the lipid A of which also contains a pEtN rather than a pyrophosphoethanolamine group at the 1-position.

BLASTp analysis shows a distant homolog (E value $<10^{-20}$) of Hp0022 in *P. gingivalis*. However, there are no significant homologs of Hp0021 (E value $<10^{-4}$ using BLASTp) in the sequenced genome of *P. gingivalis* W83. This is not too surprising considering Hp0021 is only distantly related to *Rhizobium* LpxE and is not detected as a *H. pylori* homolog using BLASTp. Therefore, further biochemical investigation will be required to determine if *P. gingivalis* uses a mechanism similar to that of *H. pylori* during the modification of its lipid A.

With our evidence that Hp0021 phosphatase activity in *E. coli* is MsbA-dependent we propose a periplasmic model for the modification of the *H. pylori* lipid A 1-phosphate group. Following synthesis of bisphosphorylated Kdo₂-lipid A via the constitutive pathway, the lipid is flipped across the inner membrane by the ABC transporter, MsbA (28,39,66-68). Hp0021, an inner membrane phosphatase then removes the proximal phosphate group from *H. pylori* lipid A. This is followed by addition of a pEtN moiety to the disaccharide backbone catalyzed by the lipid A pEtN transferase, Hp0022. It is very likely that the glycerophospholipid phosphatidylethanolamine, PtdEtn, serves as the donor substrate since *E. coli* that are unable to synthesize PtdEtn are unable to modify their lipid A with pEtN residues (69). On the whole,
modification of the lipid A structure in the extracytoplasmic compartments has become a theme in the biosynthesis of lipid A given that a number of lipid A modifying enzymes are outer membrane proteins (30,40) or have active sites facing the periplasmic region (55,69-72).

Our work has corroborated the structure previously reported for *H. pylori* lipid A (13-15). We are currently in the process of constructing *H. pylori* mutants lacking functional copies of *hp0021* and *hp0022*. These mutants will help us determine the importance of pEtN addition to the C-1 hydroxyl group of *H. pylori* lipid A during infection. Also, since none of the *H. pylori* PgpB orthologs showed lipid A 4′-phosphatase activity, we are actively trying to identify the structural gene encoding this enzyme. Changing the charge of the lipid A domain of *H. pylori* LPS most likely aids the bacterium in establishing the prolonged, chronic infections seen with this organism by providing resistance to cationic antimicrobial peptides and reducing the endotoxic principle of *H. pylori* LPS.
Figure legends

Figure 1. Comparison of the chemical structures of the Kdo-lipid A domain of *E. coli* and the major and minor structures of *H. pylori*. The covalent modifications of lipid A are indicated with dashed bonds, and lengths of the acyl chains are designated with enclosed circles.

**Panel A.** The major lipid A species of *E. coli* is a hexa-acylated disaccharide of glucosamine that is substituted at the 1- and 4'-positions with phosphate and glycosylated at the 6' position with two Kdo (3-deoxy-α-manno-octulosonic acid) moieties (1,5). In wild type K12 strains, a portion of the lipid A contains a pyrophosphate group at the 1-position (73). The modification of phosphate moieties of *E. coli* lipid A are regulated by PmrA (74) and may be substituted with L-Ara4N (red substituent) or pEtN (pink substituent) groups. In addition, the protein product of the PhoP-activated gene, *pagP*, further modifies *E. coli* lipid A by the addition of a palmitoyl chain (blue substituent) to the hydroxyl group of the N-linked-R-3-hydroxymyristate chain on the proximal glucosamine unit of lipid A (40,44). **Panel B.** The minor lipid A species of *H. pylori* is similar to that seen in *E. coli* but has slightly longer acyl chains and only contains one Kdo moiety (13,14,19). **Panel C.** The major lipid A species of *H. pylori* is a tri- to tetra-acylated structure that is lacking the 4'-phosphate group and is substituted at the C-1 position with a pEtN residue (13-15).

Figure 2. Detection of 1-phosphatase activity in membranes of wild type strains of *H. pylori*. Membranes from either *E. coli* wild type strain W3110 or *H. pylori* wild type strains (26695 and J99) were assayed for 1-phosphatase activity using assay conditions described under “Experimental Procedures.” The protein concentration was 1.0 mg/ml, and assays were carried
out for both 30 min (Panel A) and 3 h (Panel B) at 30 °C. Each reaction contained 5 µM [4′-\textsuperscript{32}P]lipid IV\textsubscript{A} substrate. Reaction products were separated by TLC and detected with PhosphorImager analysis. Removal of the 1-phosphate group was indicated by conversion of the [4′-\textsuperscript{32}P]lipid IV\textsubscript{A} substrate to a faster migrating species (26).

Figure 3. Assay for 1-phosphatase activity from \textit{E. coli} membranes over-expressing putative \textit{H. pylori} PgpB homologues (Hp0021, Hp1580, Hp0851, Hp0350). Membranes from \textit{E. coli} K-12 strain, NovaBlue (DE3), containing either the vector control, or the indicated hybrid plasmids were assayed for 1-phosphatase activity using [4′-\textsuperscript{32}P]lipid IV\textsubscript{A}. The protein concentration was 0.05 mg/ml and assays were carried out for 1 hr at 30 °C with 5 µM [4′-\textsuperscript{32}P]lipid IV\textsubscript{A} substrate. The products were separated by TLC and detected with PhosphorImager analysis.

Figure 4. Substrate specificity and time dependence of the \textit{H. pylori} lipid A 1-phosphatase. Panel A. The 1-phosphatase activity of NovaBlue (DE3) membranes expressing Hp0021 was assayed under standard conditions described under “Experimental Procedures” with either 5 µM [4′\textsuperscript{32}P]lipid IV\textsubscript{A}, Kdo\textsubscript{2}-[4′\textsuperscript{32}P]lipid IV\textsubscript{A}, or Kdo\textsubscript{2}-[4′\textsuperscript{32}P]lipid A. Protein concentrations at 0.05 mg/ml from NovaBlue (DE3)/pHp0021 membranes served as the enzyme source. Reaction products were separated by TLC as described under “Experimental Procedures” and subjected to PhosphorImager analysis. Panel B. The 1-phosphatase activity of Hp0021 was assayed under standard conditions with 5 µM [4′\textsuperscript{32}P]lipid IV\textsubscript{A}. Membranes from NovaBlue (DE3) expressing Hp0021 at the indicated concentrations served as the enzyme source. Reaction products were separated by TLC as described under “Experimental Procedures” and subjected to
PhosphorImager analysis.

**Figure 5. Lipid A composition in E. coli K-12 strains expressing putative H. pylori PgpB orthologs.** The $^{32}$P-Labeled lipid A species from HMS174 (DE3) containing the vector control (pETcoco-2), or the indicated hybrid plasmids were isolated as described under “Experimental Procedures.” The labeled lipid A species were separated by TLC using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v), and visualized by PhosphorImaging. The presence of 1-dephosphorylated-lipid A from HMS174 (DE3) expressing Hp0021 is indicated. A small amount of 1-dephosphorylated-lipid A may arise as a decomposition product during hydrolysis of the Kdo linkage at pH 4.5 at 100 °C (16, 17, 36). A reduced amount of $^{32}$P-labeled lipid A was isolated from bacteria expressing Hp0851 and Hp0350 due to toxic effects upon protein expression.

**Figure 6. MALDI/TOF mass spectrometry of the 1-phosphatase reaction product generated from Kdo$_2$-Lipid A.** The reaction product generated from incubation of hexa-acylated Kdo$_2$-lipid A with NovaBlue (DE3)/pHp0021 membranes was purified by DEAE cellulose chromatography as described under “Experimental Procedures.” The spectra shown in Panel A is that of the starting Kdo$_2$-lipid A substrate and the spectra shown in Panel B shows the resulting 1-dephosphorylated reaction product, 1-dephosphorylated-Kdo$_2$-lipid A. Both spectra were acquired in the negative ion mode.

**Figure 7. Dual expression of Hp0021 and Hp0022 in E. coli results in the formation of 1-pEtN-lipid A species.** The $^{32}$P-labeled lipid A species from the indicated strains were isolated as
described under “Experimental Procedures,” separated by TLC using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v), and visualized by PhosphorImaging. A small amount of 1-dephosphorylated-lipid A may arise as a decomposition product during hydrolysis of the Kdo linkage at pH 4.5 at 100 °C (16, 17, 36). The structure of the lipid A species labeled as 1-pEtN-Lipid A was verified by mass spectrometry (Fig. 9, panel D).

**Figure 8.** MALDI/TOF mass spectrometry of lipid A from *E. coli* expressing the *H. pylori* lipid A 1-phosphatase and pEtN transferase genes. The lipid A of *E. coli* NovaBlue (DE3) containing either pDuet-1 (Panel A), pHp0021duet (Panel B), pHp0022duet (Panel C), or pHp0021/Hp0022 (Panel D) was isolated and fractionated by DEAE cellulose chromatography using published protocols (38). The lipid A samples were analyzed by MALDI/TOF mass spectrometry in the negative-ion mode. Although mass spectrometry data was acquired on lipids eluting in all salt fractions from the DEAE column, only the most representative data is shown for comparison. Data shown in Panels A and C is from lipid A species found to elute in the 240 mM salt fraction, whereas data shown in Panels B and D is from lipids eluting in the 60 mM salt fraction (see “Experimental Procedures”). The molecular weights of *E. coli* hexa-acylated bis-phosphate lipid A, 1-dephosphorylated-lipid A, and 1-pEtN-lipid A are 1798.4, 1719.4, and 1841.4 respectively. Panel E shows the positive ion spectrum of lipid A obtained from NovaBlue (DE3) containing pHp0021duet.

**Figure 9.** Removal of the lipid A 1-phosphate group by Hp0021 is dependent upon the lipid A transporter, MsbA. *E. coli* strains WD2 and W3110A containing either pWSK29 or pWSHp0021 were temperature shifted to 44 °C for 30 min. Newly synthesized lipids were ³²P-
labeled for 20 min following temperature shift and the lipid A isolated as described under ‘Experimental Procedures.’ Lipid A species from the indicated strains were separated by TLC using the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, v/v), and visualized by PhosphorImaging.

Figure 10. Proposed topology of the cleavage and modification of the 1-phosphate group of \textit{H. pylori} lipid A. After assembly of Kdo$_2$-lipid A bearing the core-oligosaccharide (core-lipid A), the macromolecule is transported across the inner membrane by the ABC transporter, MsbA (28,67). In \textit{H. pylori}, the 1-phosphate group of the lipid A domain can then be removed by a specific phosphatase Hp0021. This is followed by the addition of a pEtN unit directly to the glucosmaine disaccharide backbone catalyzed by the lipid A pEtN transferase, Hp0022. The predicted membrane topology of Hp0021 and Hp0022 was determined using the program SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) (75,76).
Footnotes

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Abbreviations

HEPES, 4-(2-hydroxyethyl)-1piperazineethanesulfonic acid; MES, 2-(N-morpholino)-ethanesulfonic acid; IPTG, iso-propyl-1-thio-β-D-galactopyranoside; Kdo, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharide; MALDI/TOF, matrix assisted laser-desorption/time of flight.
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| Strain or Plasmid | Genotype or Description | Source or Reference |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| *Helicobacter pylori* |                         |                     |
| J99               | Wild type               | ATCC 700824         |
| 26695             | Wild type               | ATCC 700392         |
| *Escherichia coli* |                         |                     |
| W3110             | Wild type, F, λ         | E. coli Genetic Stock Center (Yale) |
| W3110A            | Wild type, F, λ, aroA::Tn10 | (71)               |
| WD2               | W3110, aroA::Tn10 msbA (A270T) | (72)               |
| XL-1 Blue         | recA1 endA1 gyrA96thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZΔM15::Tn10 (TetR)] | Stratagene |
| NovaF-            | F', endA1 hsdR17(rK12 mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac | Novagen |
| NovaBlue(DE3)     | endA1 hsdR17(rK12 mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac (DE3) [F' proAB lacIqZΔM15::Tn10 (TetR)] | Novagen |
| HSM174(DE3)       | F' recA1 hsdR (rK12 mK12+) (DE3) RifR | Novagen |
| BLR(DE3)pLysS     | F' ompT hsdS6 (rB mB) gal dcm (DE3) Δ(srl-recA)306:: Tn10 pLysS, (TetR, CamR) | Novagen |
| **Plasmids**      |                         |                     |
| pET21a (+)        | Expression Vector containing a T7 promoter, AmpR | Novagen |
| pETCoco-2         | Expression vector containing a T7 promoter, and two origins of replication control the single-copy (oriS) and medium-copy (oriV), AmpR | Novagen |
| pETDuet-1         | Expression vector containing two multiple cloning sites (MCS), each of which is preceded by a T7lac promoter, lacI, AmpR | Novagen |
| pHp0021           | pET21a (+) containing hp0021 | This work |
| pHp0350           | pET21a (+) containing hp0350 | This work |
| pHp0851           | pET21a (+) containing hp0851 | This work |
| pHp1580           | pET21a (+) containing hp1580 | This work |
| pHp0022           | pET21a (+) containing hp0022 | This work |
| pHp0021coco       | pETCoco-2 containing hp0021 | This work |
| Vector | Description                                                | Source          |
|--------|-------------------------------------------------------------|-----------------|
| pHp0350coco | pETCoco-2 containing *hp0350*                           | This work       |
| pHp0851coco | pETCoco-2 containing *hp0851*                           | This work       |
| pHp1580coco | pETCoco-2 containing *hp1580*                           | This work       |
| pHp0021Duet | pETDuet-1 containing *hp0021*                          | This work       |
| pHp0022duet | pETDuet-1 containing *hp0022*                          | This work       |
| pHp0021/Hp0022 | pETDuet-1 containing both *hp0021* and *hp0022*       | This work       |
| pWSK29 | Low-copy expression vector, Amp'                      | (77)            |
| pWSHp0021 | pWSK29 containing *hp0021*                                | This work       |
| pMsbB | pET21a (+) containing *msbB (lpxL)*                    | (37)            |
| pHtrB | pET21a (+) containing *htrB (lpxM)*                     | This work       |
| Name            | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| U-hp0022-NdeI   | 5’-GCGCGCCATATGTTGGCATCATTATTCCAT-3’                                    |
| L-hp0022-BamHI  | 5’-GCGCGCGGATCTTTACTCTTTTTTGTGTTT-3’                                   |
| U-hp0021-NdeI   | 5’-GCGCGCCATATGAAAAAATTCTTATTTAAA-3’                                   |
| L-hp0221-BamHI  | 5’-GCGCGCGGATCCTTAAGGCTTTTTGGGGCT-3’                                   |
| U-hp0350-NdeI   | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp0350-BamHI  | 5’-GCGCGCGGATCCTTAGTTTTAAGGCG-3’                                       |
| U-hp0851-NdeI   | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp0851-BamHI  | 5’-GCGCGCGGATCCTTAGTTTTAAGGCG-3’                                       |
| U-hp1580-NdeI   | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp1580-BamHI  | 5’-GCGCGCGGATCCTTAGTTTTAAGGCG-3’                                       |
| U-hp0021coco-NsiI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp0021coco-BstBI | 5’-GCGCGCGGATCCTTAGTTTTAAGGCG-3’                                      |
| U-hp0350coco-NsiI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp0350coco-BstBI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| U-hp0851coco-NsiI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp0851coco-BstBI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| U-hp1580coco-NsiI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| L-hp1580coco-BstBI | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| **htrBUpper**   | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
| **htrBLower**   | 5’-GCGCGCCATATGCTTATTTGACAGAACAATC-3’                                  |
A

1 2 3 4
No Enzyme W3110 26695 J99

30 mins

B

5 6 7
W3110 26695 J99

3 hrs

Solvent Front

1-Dephospha-
[4'-32P]-Lipid IV\textsubscript{A}

Lipid IV\textsubscript{B}

[4'-32P]-Lipid IV\textsubscript{A}

Origin
pDuet-1

Lipid A
1-pyrophosphate

1-Dephospho-Lipid A

Lipid A
1,4'-bis-phosphate

Solvent Front

Origin

1 2 3 4
1-pDuet-1 phP0021duet phP0022duet phP0021/Hp0022

1-Dephospho-Lipid A

Lipid A
1,4'-bis-phosphate

1-pEtN-Lipid A
Inner Membrane

Periplasm

1-Dephospho-Kdo₂-Lipid A

MsbA

Core-Lipid A

Cytoplasm

1-pEtN-Kdo₂-Lipid A

P = phosphate

pEtN = phosphoethanolamine
Periplasmic cleavage and modification of the 1-phosphate group of helicobacter pylori lipid A
An X. Tran, Mark J. Karbarz, Xiaoyuan Wang, Christian R. Raetz, Sarah C. McGrath,
Robert J. Cotter and M. Stephen Trent

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