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Periplasmic Cleavage and Modification of the 1-Phosphate Group of *Helicobacter pylori* Lipid A*

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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 disaccharide 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. By 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 *Escherichia 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 shows 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.

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),[1] 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 3-deoxy-D-*manno*-octulosonic acid (Kdo) 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 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 bisphosphorylated and multiply acylated. In *Escherichia 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. 1*A*) (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 *Helicobacter 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. 1*C*) (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 with 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 bisphosphorylated and hexa-acylated (Fig. 1*B*) resembling entero-

[1] The abbreviations used are: LPS, lipopolysaccharide; pEtN, phos-

phoethanolamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; IPTG, iso-propyl-1-thio-β-D-galactopyranoside; Kdo, 3-deoxy-D-*manno*-octulosonic acid; MALDI-TOF, matrix assisted laser-desorption/time of flight.
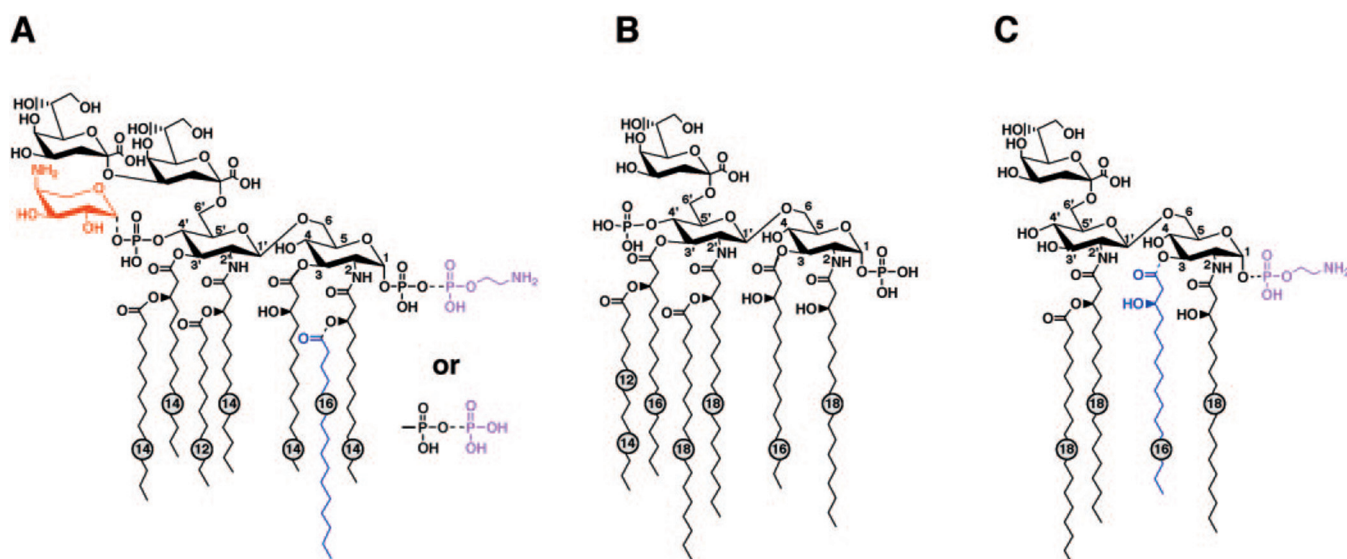
FIG. 1. **Comparison of the chemical structures of the Kdo-lipid A domain of *E. coli* and the major and minor structures of *H. pylori*.** The covalent modifications of lipid A are indicated with *dashed bonds*, and lengths of the acyl chains are designated with *numbers in circles. A*, the major lipid 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-D-*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 (*purple* 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). *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). *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).

bacterial 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. 1*A*. 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-^{32}P]ATP and ^{32}P$_i$ were obtained from Amersham Biosciences. 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% O$_2$, 10% CO$_2$, and 85% N$_2$). The resultant colonies were inoculated in *Brucella* broth (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and vancomycin (10 μg/ml). Cells were grown to an A$_{600}$ 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* was 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 by 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 manufacturer's 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 *N. meningitidis* lptA (60) (hp0022) were cloned into pET21a(+) behind the T7*lac* 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 pgpB* and *lptA* orthologs are described in Table II. The forward primers contained a clamp region, an NdeI site (Table II, 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 (Table II, underlined), and a match to the anti-coding strand that included a stop codon. The PCR 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 MgCl$_2$, 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 vectors 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—The *H. pylori* PgpB and LptA ortholog constructs derived from pET21a(+) were transformed into NovaBlue(DE3) (Table I) for overexpression of the protein. First, a single colony of *E. coli* harboring either the empty vector control or the recombinant expression plasmid were inoculated into 50 ml of LB broth and grown overnight (~16 h) in a rotary shaker at 37 °C to A$_{600}$ ~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 1 mM IPTG, cells were allowed to reach mid-log phase of growth (A$_{600}$ ~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-PAGE, 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

TABLE I
Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or Reference
Strains		
H. pylori		
J99	Wild type	ATCC 700824
26695	Wild type	ATCC 700392
E. 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* (Tet^R)]	Stratagene
Nova F−	F⁻, *endA1 hsdR17*(r$_{K12}$⁻ m$_{K12}$+) *supE44 thi-1 recA1 gyrA96 relA1 lac*	Novagen
NovaBlue(DE3)	*endA1 hsdR17*(r$_{K12}$⁻ m$_{K12}$+) *supE44 thi-1 recA1 gyrA96 relA1 lac* (DE3) [F′ *proAB lacIqZΔM15::Tn10* (Tet^R)]	Novagen
HSM174(DE3)	F⁻ *recA1 hsdR* (r$_{K12}$⁻ m$_{K12}$+) (DE3) Rif ^{R}	Novagen
BLR(DE3)pLysS	F⁻ *ompT hsdS$_B$* (r$_B$⁻ m$_B$⁻) *gal dcm* (DE3) Δ(*srl-recA*)306:: Tn*10* pLysS, (Tet^R, Cam^R)	Novagen
Plasmids		
pET21a(+)	Expression vector containing a T7 promoter, Amp^R	Novagen
pETCoco-2	Expression vector containing a T7 promoter, and two origins of replication control the single-copy (*oriS*) and medium-copy (*oriV*), Amp^R	Novagen
pETDuet-1	Expression vector containing two multiple cloning sites (MCS), each of which is preceded by a T7lac promoter, lacI, Amp^R	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
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^r	77
pWSHp0021	pWSK29 containing *hp0021*	This work
pMsbB	pET21a (+) containing *msbB* (*lpxL*)	37
pHtrB	pET21a (+) containing *htrB* (*lpxM*)	This work

TABLE II
Oligonucleotides

Name	Sequence
U-*hp0022*-NdeI	5′-GCGCGC<u>CATATG</u>TTGGCATCATTATTCCAT-3′
L-*hp0022*-BamHI	5′-GCGCGC<u>GGATCC</u>TTACTCTTTTTTGTGTTT-3′
U-*hp0021*-NdeI	5′-GCGCGC<u>CATATG</u>AAAAAATTCTTATTTAAA-3′
L-*hp0221*-BamHI	5′-GCGCGC<u>GGATCC</u>TTAAGGCTTTTTGGGGCT-3′
U-*hp0350*-NdeI	5′-GCGCGC<u>CATATG</u>CTGAAAATTCTTTCAAA-3′
L-*hp0350*-BamHI	5′-GCGCGC<u>GGATCC</u>TTAGATCTTTTGAAACAC-3′
U-*hp0851*-NdeI	5′-GCGCGC<u>CATATG</u>TATTTGACAGAACAATC-3′
L-*hp0851*-BamHI	5′-GCGCGC<u>GGATCC</u>TTATTGATTATAAGGGCG-3′
U-*hp1580*-NdeI	5′-GCGCGC<u>CATATG</u>TCTATTTAATGGGCTATGC-3′
L-*hp1580*-BamHI	5′-GCGCGC<u>GGATCC</u>TTACCATTGATAAGAAAA-3′
U-*hp0021*coco-NsiI	5′-GCGCGC<u>CATGCA</u>TAAAAAATTCTTATTTAAA-3′
L-*hp0021*coco-BstBI	5′-GCGCGC<u>CTTCGA</u>ATTAAGGCTTTTTGGGGCT-3′
U-*hp0350*coco-NsiI	5′-GCGCGC<u>CATGCA</u>TGCTGAAAATTCTTTCAAA-3′
L-*hp0350*coco-BstBI	5′-GCGCGC<u>CTTCGA</u>ATTAGATCTTTTGAAACAC-3′
U-*hp0851*coco-NsiI	5′-GCGCGC<u>CATGCA</u>TGTATTTGACAGAACAATC-3′
L-*hp0851*coco-BstBI	5′-GCGCGC<u>CTTCGA</u>ATTATTGATTATAAGGGCG-3′
U-*hp1580*coco-NsiI	5′-GCGCGC<u>CATGCA</u>TCATTATTTAATGGGCTATGC-3′
L-*hp1580*coco-BstBI	5′-GCGCGC<u>CTTCGA</u>ATTACCATTGATAAGAAAA-3′
*htrB*Upper	5′-GCGCGC<u>CATATG</u>ATGACGAATCTACCC-3′
*htrB*Lower	5′-GCGGCG<u>GGATCC</u>TTAATAGCGTGAAGG-3′

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 NsiI site (Table II, 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 BstBI site (Table II, 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 NsiI and BstBI, gel-purified, and ligated together. Plasmid constructs derived from pET-Coco-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 μg/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 was 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 because 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 h prior to harvest for plasmid purification. Plasmid constructs were then screened for positive inserts, confirmed by DNA sequencing, and transformed into *E. coli* K12 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 1 of pETDuet-1 to derive the plasmid construct pHp0021duet. In addition, the plasmid constructs pHp0022, pET-Duet-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 described previously.

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 overexpressed as described previously (29). Primer sequences for *lpxL* are described in Table II. The *lpxL* PCR product was digested with NdeI 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_{600} 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_{600} of 1.0 at 37 °C and harvested by centrifugation at 6,000 × g for 30 min. All samples were prepared at 4 °C. Cell-free extract, double-spun cytosol, and washed membranes were prepared as described previously (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′-^{32}P-lipid IV$_A$ was generated from 100 μCi of [γ-^{32}P]ATP and the tetraacyl-disaccharide 1-phosphate lipid acceptor, using the overexpressed 4′-kinase present in membranes of *E. coli* BLR(DE3)/pLysS/pJK2 (30, 32, 33), as described previously (30, 33). Kdo$_2$-4′-^{32}P-lipid IV$_A$ was prepared by adding purified *E. coli* Kdo transferase (WaaA) immediately after the 4′-kinase, as described previously (33, 34). To prepare Kdo$_2$-4′-^{32}P-lipid A (dilauroyl-Kdo$_2$-4′-^{32}P-lipid IV$_A$), lauroyl-acyl carrier protein 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$_2$, 0.1 mg/ml bovine serum albumin, 13.3 μM lauroyl-acyl carrier protein, and 0.05 mg/ml BLR(DE3)/pLysS/pMsbB and BLR(DE3)/pLysS/pHtrB membranes. Once the reaction was complete, the substrate was isolated as described previously (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′-^{32}P-lipid IV$_A$, Kdo$_2$-4′-^{32}P-lipid IV$_A$, or Kdo$_2$-4′-^{32}P-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 was dried under a cool air stream for 20 min.

When 4′-^{32}P-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′-^{32}P-lipid IV$_A$ or Kdo$_2$-4′-^{32}P-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 onto a seven-step gradient, as described previously (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 described previously (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 Nova-Blue(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} ~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 × 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 described previously (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 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 system consisting of chloroform, methanol, 0.05 M HCl (2:2:1.8, v/v). The phases were separated by centrifugation at 4000 × 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), 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 then charring. All fractions containing 1-dephosphorylated-Kdo$_2$-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 N$_2$ 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 M_r ~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.
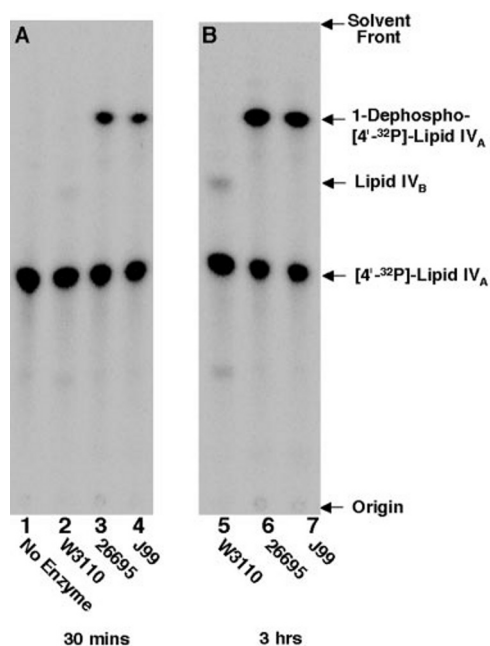
FIG. 2. **Detection of 1-phosphatase activity in membranes of wild type strains of H. pylori.** Membranes from either *E. coli* wild type strain W3110 or *H. pylori* wild type strains (26695 and J99) were assayed for 1-phosphatase activity using assay conditions described under "Experimental Procedures." The protein concentration was 1.0 mg/ml, and assays were carried out for both 30 min (*A*) and 3 h (*B*) at 30 °C. Each reaction contained 5 μM $4'$-^{32}P-lipid IV$_A$ substrate. Reaction products were separated by TLC and detected with PhosphorImager analysis. Removal of the 1-phosphate group was indicated by conversion of the $4'$-^{32}P-lipid IV$_A$ substrate to a faster migrating species (26).

FIG. 3. **Assay for 1-phosphatase activity from E. coli membranes overexpressing putative H. pylori PgpB homologs (Hp0021, Hp1580, Hp0851, and Hp0350).** Membranes from *E. coli* K12 strain, NovaBlue(DE3), containing either the vector control or the indicated hybrid plasmids were assayed for 1-phosphatase activity using $4'$-^{32}P-lipid IV$_A$. The protein concentration was 0.05 mg/ml, and assays were carried out for 1 h at 30 °C with 5 μM $4'$-^{32}P-lipid IV$_A$ substrate. The products were separated by TLC and detected with PhosphorImager 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 $4'$-^{32}P-lipid IV$_A$, 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-$4'$-^{32}P-lipid IV$_A$ (Fig. 2, *lanes 3, 4, 6,* and *7*). Removal of the 1-phosphate group from $4'$-^{32}P-lipid IV$_A$ increases the hydrophobicity of the lipid substrate, resulting in a faster migrating lipid species when analyzed by TLC in the solvent chloroform, pyridine, 88% formic acid, and water (50:50:16:5, v/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 ^{32}P$_i$ from the $4'$-position of lipid A when analyzed by TLC. However, a slower migrating species, lipid IV$_B$, was detected from membranes of wild type *E. coli* strain W3110 after 3 h at 30 °C (Fig. 2, *lane 5*). Lipid IV$_B$ is generated by the addition of a palmitate chain to the amide-linked β-hydroxmyristate residue at position 2 of lipid IV$_A$ (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, *lanes 2* and *5*).

Identification of the H. pylori 1-Phosphatase (Hp0021) from Putative Orthologs of E. coli PgpB—Recently, Karbarz *et al.* (46) identified LpxE, a phosphatase that selectively removes the 1-phosphate group of *Rhizobium 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 data base (49–52) using *E. coli* PgpB to identify possible *H. pylori*
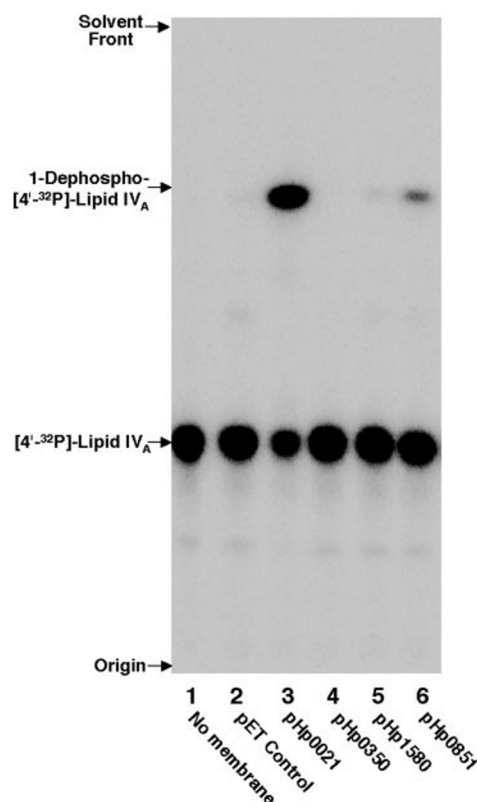
lipid phosphatases. COG0671 contains four putative orthologs of PgpB in *H. pylori* strain 26695*,* designated Hp0021, Hp0350, Hp0851, and Hp1580. The *H. pylori* PgpB orthologs were examined for 1-phosphatase activity by expressing the putative lipid phosphatases in the *E. coli* K12 strain NovaBlue(DE3). Membranes isolated from NovaBlue(DE3) containing either pET21a(+) (vector control), pHp0021, pHp1580, pHp0851, or pHp0350 were assayed with $4'$-^{32}P-lipid IV$_A$ 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, *lanes 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 nonspecific (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 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. 4*A*) and does not require the Kdo moiety or complete acylation for activity, as indicated by
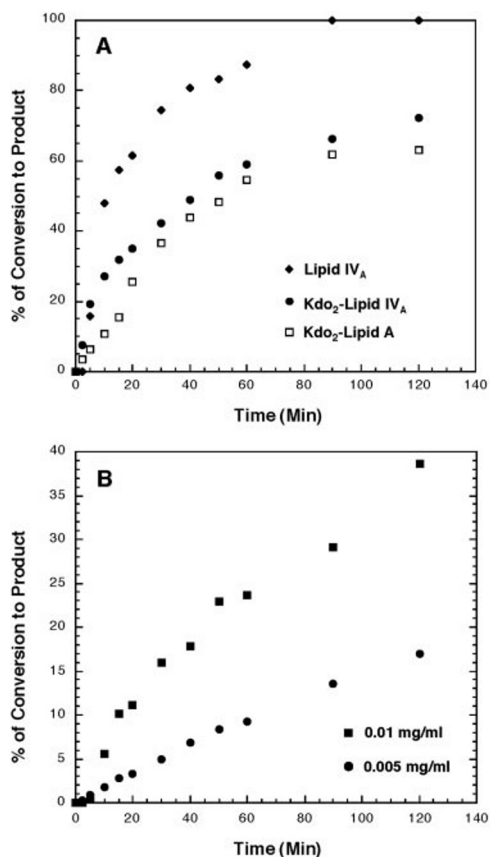
FIG. 4. **Substrate specificity and time dependence of the *H. pylori* lipid A 1-phosphatase.** *A*, the 1-phosphatase activity of NovaBlue(DE3) membranes expressing Hp0021 was assayed under standard conditions described under "Experimental Procedures" with either 5 μM 4' ^{32}P-lipid IV$_A$, Kdo$_2$-4' ^{32}P-lipid IV$_A$, or Kdo$_2$-4' ^{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. *B*, the 1-phosphatase activity of Hp0021 was assayed under standard conditions with 5 μM 4' ^{32}P-lipid IV$_A$. 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.

the higher amount of activity with 4'-^{32}P-lipid IV$_A$ than with either Kdo$_2$-4'-^{32}P-lipid IV$_A$ or Kdo$_2$-4'-^{32}P-lipid A. Formation of the 1-dephosphorylated reaction products is linear with both protein concentration and time. As shown in Fig. 4*B*, the 1-de-phosphorylation of 5 μM 4'-^{32}P-lipid IV$_A$ by 0.01 mg/ml Nova-Blue(DE3)/pHp0021 membranes is linear with time for ~40 min at 30 °C. Next, the complete 1-dephosphorylation of 5 μM 4'-^{32}P-lipid IV$_A$ is seen after 1.5 h when using 0.05 mg/ml of membrane protein (Fig. 4*A*). Overall, the *H. pylori* lipid A 1-phosphatase is a very stable enzyme having a specific activity of ~170 nmol/min/mg with lipid IV$_A$ as the substrate and *E. coli* membranes overexpressing 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 nonspecific, 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, pHp0851c-oco, and pHp0350coco. A low copy expression system was utilized
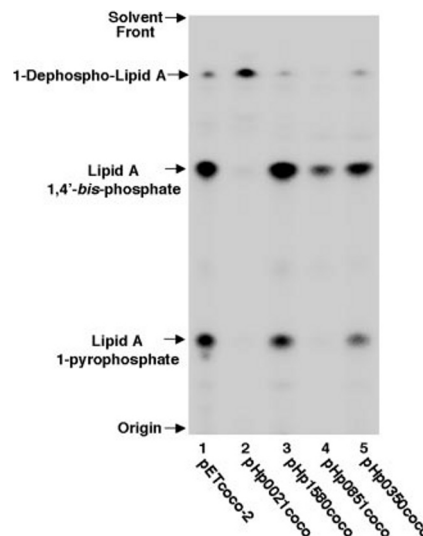
FIG. 5. **Lipid A composition in *E. coli* K12 strains expressing putative *H. pylori* PgpB orthologs.** The ^{32}P-labeled lipid A species from HMS174(DE3) containing the vector control (pETCoco-2) or the indicated hybrid plasmids were isolated as described under "Experimental Procedures." The labeled 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-de-phosphorylated-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 because of toxic effects upon protein expression.

to avoid nonspecific phosphatase activity generated from high levels of protein expression. Cultures of the *E. coli* K12 strain HMS174(DE3) containing one of the pETCoco-2 derived plasmids were labeled with ^{32}P$_i$, and the lipid A fraction was isolated to determine possible modifications *in vivo*.

HMS174(DE3) containing the pETCoco-2 vector control produced the typical ^{32}P-labeled lipid A species seen in wild type *E. coli* K12, 1,4'-bisphosphate 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. ^{32}P-Labeled lipid A fractions isolated from cells containing pHp0350coco, pHp0851coco, and pHp1580coco did not contain any significant amount of 1-dephosphorylated lipid A species (Fig. 5, *lanes 3–5*). The minor amount of 1-dephosphorylated lipid seen in Fig. 5, *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 ^{32}P-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 Kdo$_2$-lipid A as the substrate was analyzed by MALDI-TOF mass spectrometry in the negative mode. MALDI-TOF
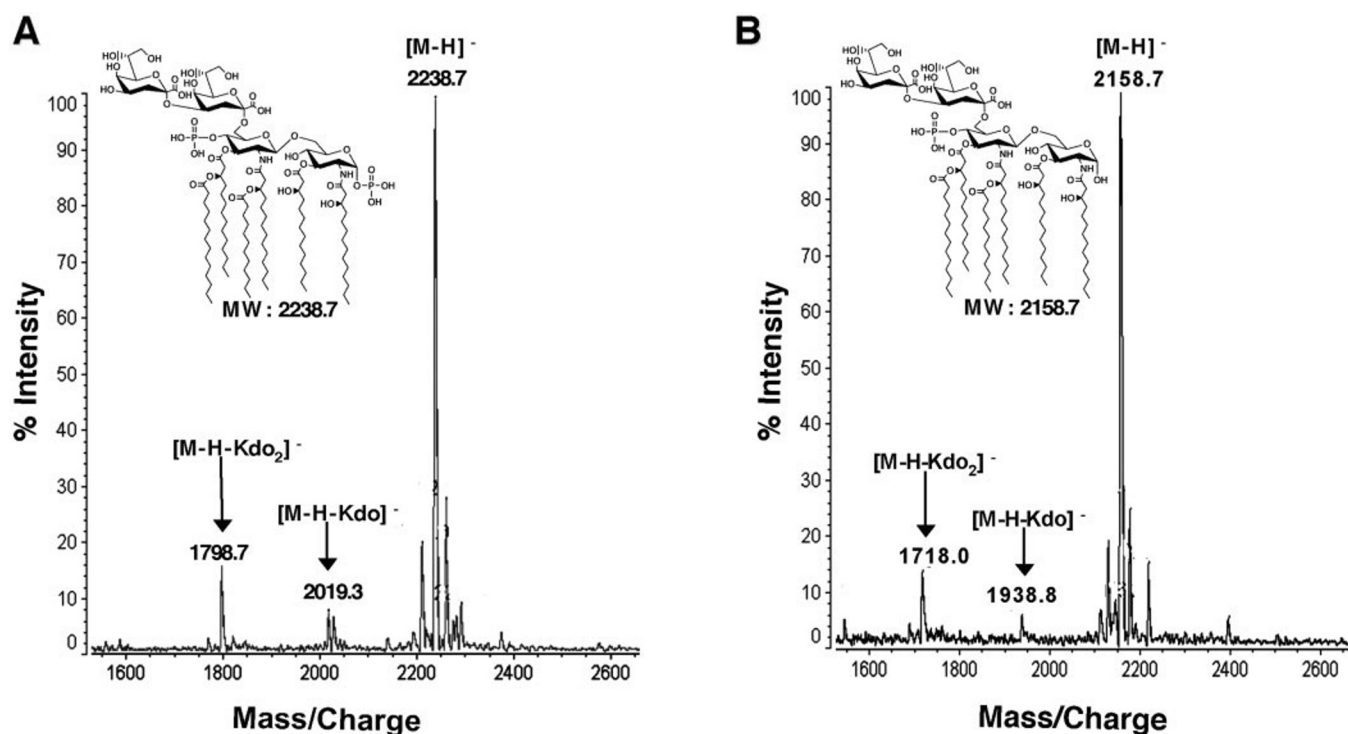
FIG. 6. **MALDI-TOF mass spectrometry of the 1-phosphatase reaction product generated from Kdo₂-lipid A.** The reaction product generated from incubation of hexa-acylated Kdo₂-lipid A with NovaBlue(DE3)/pHp0021 membranes was purified by DEAE-cellulose chromatography as described under "Experimental Procedures." The spectra shown in *A* is that of the starting Kdo₂-lipid A substrate, and the spectra shown in *B* shows the resulting 1-dephosphorylated reaction product, 1-dephosphorylated-Kdo₂-lipid A. Both spectra were acquired in the negative ion mode.

mass spectrometry of the initial substrate *E. coli* Kdo$_2$-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*A*). The signal at m/z 2238.7 atomic mass units is interpreted as $[M - H]^-$ of the predominant hexa-acylated Kdo$_2$-lipid A 1,4′-bisphosphate molecule (Fig. 6*A*), characteristic of *E. coli* K12. The peaks at m/z 2019.3 and 1798.7 atomic mass units are interpreted as $[M - H-Kdo]^-$ and $[M - H-Kdo_2]^-$, respectively, and correspond to the loss of the labile Kdo moiety during mass spectrometry (Fig. 6*A*). MALDI-TOF mass spectrometry of the Hp0021 reaction product revealed a predominant peak at m/z 2158.4 atomic mass units confirming the enzymatic removal of the 1-phosphate group (Fig. 6*B*) from the Kdo$_2$-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. 6*B*).

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. 1*C*). 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.* (53) identified NMB1638, renamed LptA, as a pEtN transferase specific for *N. meningitidis* lipid A based upon loss of pEtN from lipid A in an *lptA* mutant. Also, it has been shown *in vitro* that *E. coli* YjdB (EptA) transfers pEtN to *E. coli* lipid A precursors (54). Examination of the COG data base using either *N. meningitidis* NMB1638 or *E. coli* YjdB 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 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′-^{32}P-lipid IV$_A$, Kdo$_2$-4′-^{32}P-lipid IV$_A$, and Kdo$_2$-4′-^{32}P-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). These 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 *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*lac* promoters by cloning *hp0021* and *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 ^{32}P-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 *E. coli* lipid A species. As demonstrated in Fig. 7, *lane 3*, ^{32}P-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 *E. coli* lipid A. ^{32}P-Labeled lipid A species isolated from Nova-Blue (DE3) containing the dual expression construct, pHp0021/

FIG. 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*D*).
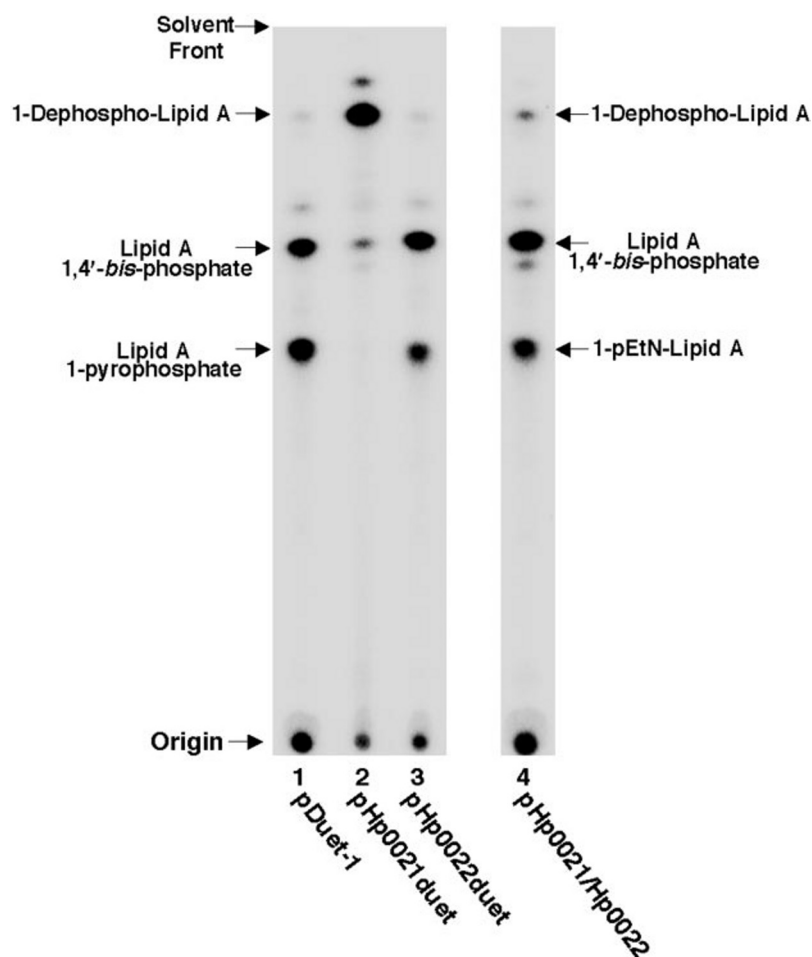
Hp0022, revealed the following three lipid species: 1,4′-bisphosphorylated lipid A, 1-dephosphorylated lipid A, and lipid A containing a pEtN group at the C-1 position (see Fig. 8). Removal of the 1-phosphate group from 1,4′-bisphosphorylated lipid A followed by the addition of 1-pEtN unit generated 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 to confirm that *hp0022* encodes for a pEtN transferase acting after 1-dephosphorylation of *H. pylori* lipid A.

The lipid A of *E. coli* strain NovaBlue(DE3) containing either pETDuet-1, pHp0021duet, pHP0022duet, 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 bisphosphate species with MALDI-TOF analysis resulting in a major peak at *m/z* 1798.4 (16, 38) (Fig. 8*A*). 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. 8*C*). As expected, expression of Hp0021 resulted in the loss of a single phosphate unit from lipid A (Fig. 8*B*), 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. 8*E*). Mass spectrometry analysis of lipid A isolated from cells overexpressing both Hp0021 and Hp0022 demonstrated two ion peaks at *m/z* 1719.0 and 1840.9 atomic mass units in the negative mode (Fig. 8*D*). 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 as follows: 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 described previously by Doerrler *et al.* (67), 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 the 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). By 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 A_{600} 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 ^{32}P$_i$ for 20 min, and the newly

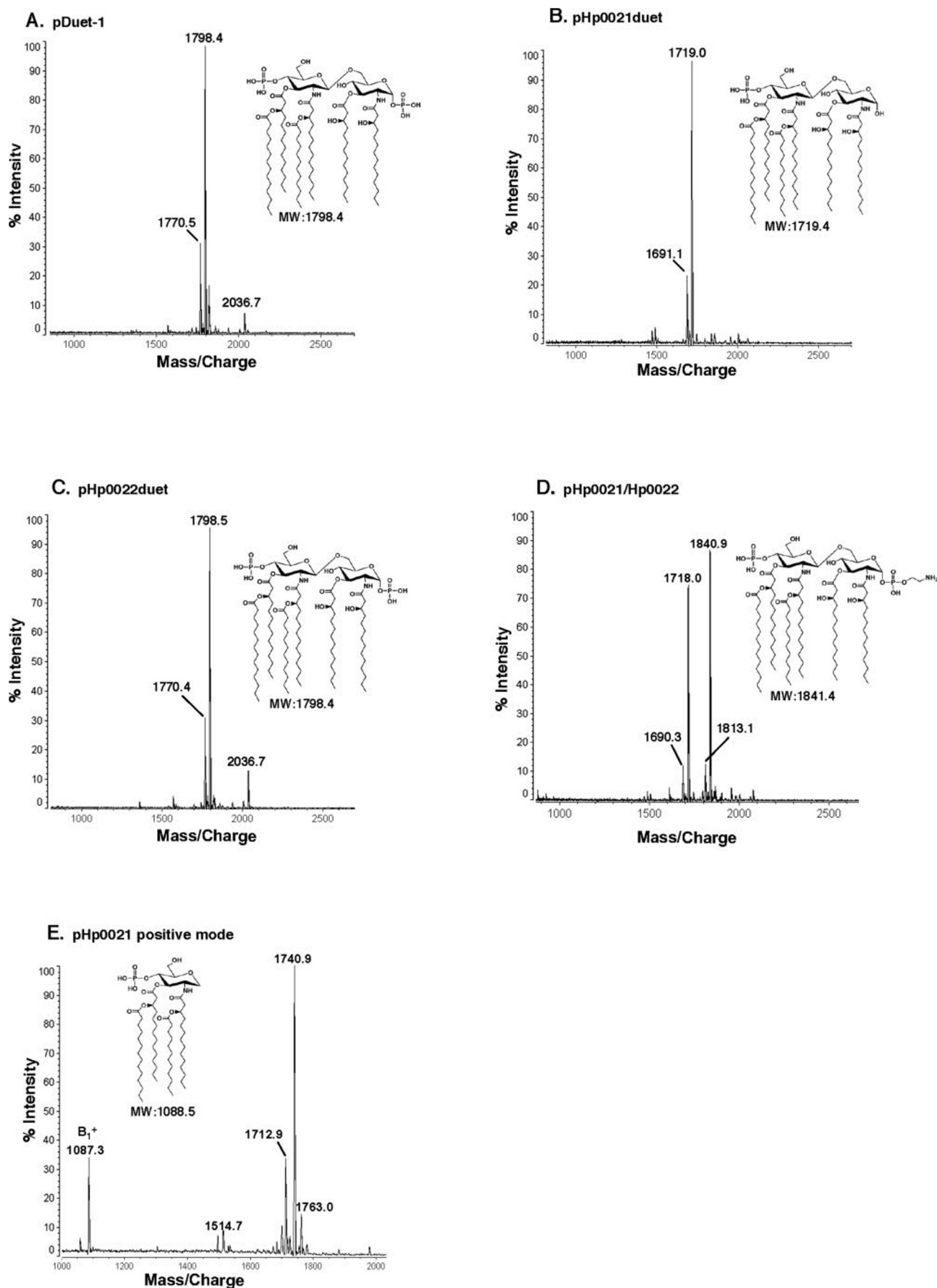FIG. 8. **MALDI-TOF mass spectrometry of lipid A from *E. coli* expressing the *H. pylori* lipid A 1-phosphatase and pEtN transferase genes.** The lipid A of *E. coli* NovaBlue(DE3) containing either pDuet-1 (*A*), pHp0021duet (*B*), pHp0022duet (*C*), or pHp0021/Hp0022 (*D*) was isolated and fractionated by DEAE-cellulose chromatography using published protocols (38). The lipid A samples were analyzed by MALDI-TOF
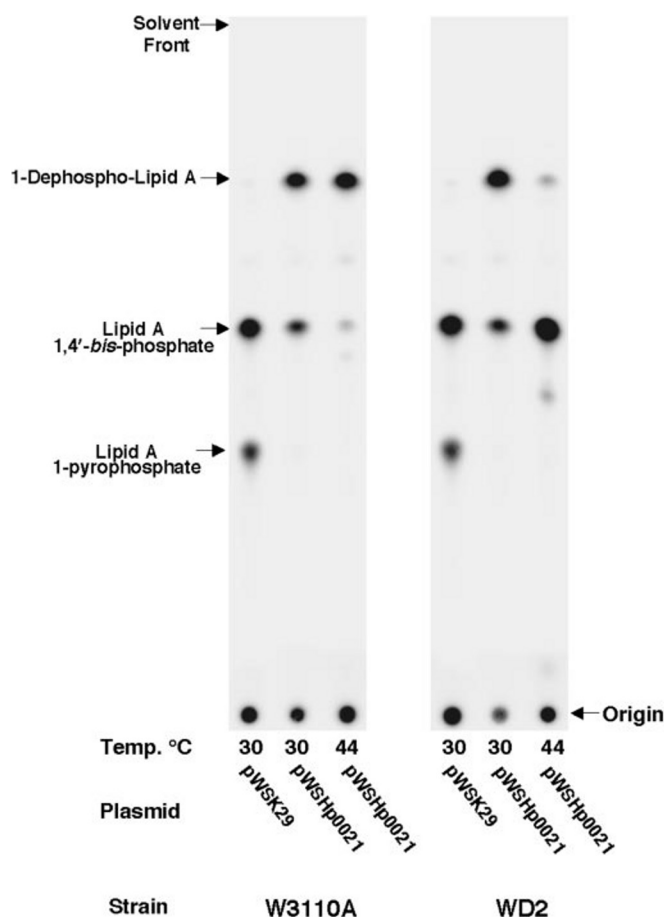
FIG. 9. **Removal of the lipid A 1-phosphate group by Hp0021 is dependent upon the lipid A transporter, MsbA.** *E. coli* strains WD2 and W3110A containing either pWSK29 or pWSHp0021 were temperature-shifted to 44 °C for 30 min. Newly synthesized lipids were ^{32}P-labeled for 20 min following temperature shift, and the lipid A was 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.

synthesized ^{32}P-labeled lipid A species was isolated and separated by TLC. At 30 °C, W3110A (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 toward the periplasm. Recently, Wang *et al.* (55) showed a similar result using a lipid A 1-phosphatase cloned from *Franciscella novicida*.

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. 1*C*) (13, 14, 19). A number of Gram-negative bacteria modify the phosphate groups of their lipid A (58).

Recently, Lee *et al.* (72) has shown that addition of pEtN to the 1-phosphate of *S. enterica* is important for resistance to the antimicrobial peptide polymyxin. In the case of *H. pylori*, the C-1 hydroxyl group of the proximal glucosamine sugar is derivatized with pEtN (13, 14, 19) (Fig. 1*C*). 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 *et al.* (46) reported the cloning of a membrane-bound enzyme, LpxE, which removes the 1-phosphate group of *R. leguminosarum* lipid A. 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 data base 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 multitransmembrane 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 Kdo_2-lipid A (Figs. 4 and 6). Membranes from wild type *H. pylori* strains 26695 and J99 catalyzed the 1-dephosphorylation of lipid IV$_A$ 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* overexpressing Hp0021 showed a specific activity of 170 nmol/min/mg resulting in more than a 100-fold increase over wild type levels. Most interestingly, expression of Hp0021 in *E. coli* K12 strains NovaBlue(DE3) (Fig. 7 and Fig. 8*B*) 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 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 because 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 sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html), Hp0022 has six transmembrane regions with the soluble C-terminal

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 *A* and *C* are from lipid A species found to elute in the 240 mM salt fraction, whereas data shown in *B* and *D* are from lipids eluting in the 60 mM salt fraction (see "Experimental Procedures"). The molecular weights of *E. coli* hexa-acylated bisphosphate lipid A, 1-dephosphorylated-lipid A, and 1-pEtN-lipid A are 1798.4, 1719.4, and 1841.4, respectively. *E* shows the positive ion spectrum of lipid A obtained from NovaBlue(DE3) containing pHp0021duet.
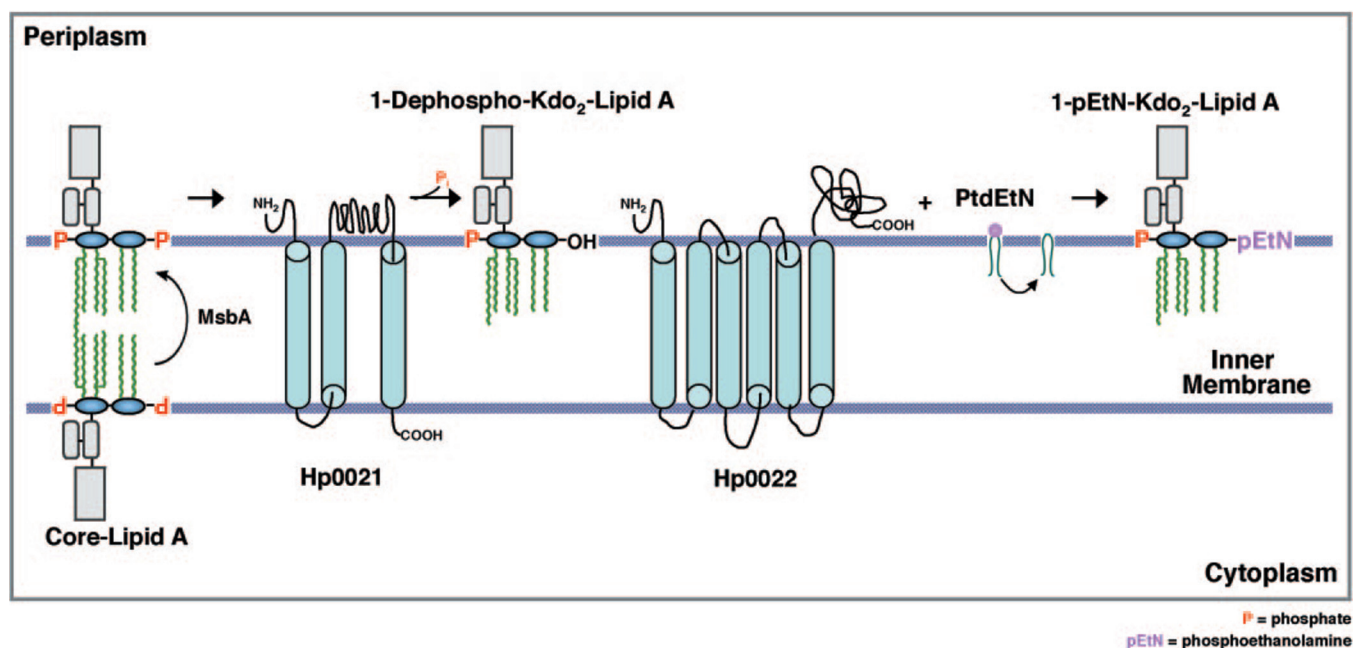
Periplasmic Addition of pEtN to H. pylori Lipid A

FIG. 10. **Proposed topology of the cleavage and modification of the 1-phosphate group of *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 *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 glucosamine disaccharide backbone catalyzed by the lipid A pEtN transferase, Hp0022. The predicted membrane topology of Hp0021 and Hp0022 was determined using the program SOSUI (sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html) (75, 76).

region predicted to be located in the periplasmic region of the cell envelope. Expression of Hp0022 alone in an *E. coli* K12 strain did not result in modification to the bisphosphorylated *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 and 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 whether *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 (Fig. 10). Following synthesis of bisphosphorylated Kdo$_2$-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 PtdEtn serves as the donor substrate because *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 reported previously 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, because 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.

REFERENCES

1. Raetz, C. R., and Whitfield, C. (2002) *Annu. Rev. Biochem.* **71,** 635–700
2. Raetz, C. R. (1990) *Annu. Rev. Biochem.* **59,** 129–170
3. Gronow, S., and Brade, H. (2001) *J. Endotoxin. Res.* **7,** 3–23
4. Galloway, S. M., and Raetz, C. R. (1990) *J. Biol. Chem.* **265,** 6394–6402
5. Raetz, C. R. H. (1996) in *Cellular and Molecular Biology* (Niedhardt, F., ed) pp. 1035–1063, American Society for Microbiology, Washington, D. C.
6. Raetz, C. R. (1993) *J. Bacteriol.* **175,** 5745–5753
7. Bone, R. C. (1993) *Clin. Microbiol. Rev.* **6,** 57–68
8. Medzhitov, R., and Janeway, C., Jr. (2000) *Trends Microbiol.* **8,** 452–456
9. Medzhitov, R., and Janeway, C., Jr. (2000) *N. Engl. J. Med.* **343,** 338–344
10. Aderem, A., and Ulevitch, R. J. (2000) *Nature* **406,** 782–787
11. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999) *J. Immunol.* **162,** 3749–3752
12. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282,** 2085–2088
13. Moran, A. P., Lindner, B., and Walsh, E. J. (1997) *J. Bacteriol.* **179,** 6453–6463
14. Suda, Y., Kim, Y. M., Ogawa, T., Yasui, N., Hasegawa, Y., Kashihara, W., Shimoyama, T., Aoyama, K., Nagata, K., Tamura, T., and Kusumoto, S. (2001) *J. Endotoxin. Res.* **7,** 95–104
15. Suda, Y., Ogawa, T., Kashihara, W., Oikawa, M., Shimoyama, T., Hayashi, T., Tamura, T., and Kusumoto, S. (1997) *J. Biochem. (Tokyo)* **121,** 1129–1133
16. Zhou, Z., Lin, S., Cotter, R. J., and Raetz, C. R. (1999) *J. Biol. Chem.* **274,** 18503–18514
17. Zhou, Z., Ribeiro, A. A., Lin, S., Cotter, R. J., Miller, S. I., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 43111–43121

18. Kulshin, V. A., Zahringer, U., Lindner, B., Frasch, C. E., Tsai, C. M., Dmitriev, B. A., and Rietschel, E. T. (1992) *J. Bacteriol.* **174,** 1793–1800
19. Ogawa, T., Asai, Y., Sakai, Y., Oikawa, M., Fukase, K., Suda, Y., Kusumoto, S., and Tamura, T. (2003) *FEMS Immunol. Med. Microbiol.* **36,** 1–7
20. Ogawa, T., Suda, Y., Kashihara, W., Hayashi, T., Shimoyama, T., Kusumoto, S., and Tamura, T. (1997) *Vaccine* **15,** 1598–1605
21. Schromm, A. B., Brandenburg, K., Loppnow, H., Moran, A. P., Koch, M. H., Rietschel, E. T., and Seydel, U. (2000) *Eur. J. Biochem.* **267,** 2008–2013
22. Schromm, A. B., Brandenburg, K., Loppnow, H., Zahringer, U., Rietschel, E. T., Carroll, S. F., Koch, M. H., Kusumoto, S., and Seydel, U. (1998) *J. Immunol.* **161,** 5464–5471
23. Appelmelk, B. J., Monteiro, M. A., Martin, S. L., Moran, A. P., and Vandenbroucke-Grauls, C. M. (2000) *Trends Microbiol.* **8,** 565–570
24. Appelmelk, B. J., Negrini, R., Moran, A. P., and Kuipers, E. J. (1997) *Trends Microbiol.* **5,** 70–73
25. Appelmelk, B. J., and Vandenbroucke-Grauls, C. M. (2000) *Gut* **47,** 10–11
26. Miller, J. R. (1972) *Experiments in Molecular Genetics,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Med. Sci.* **37,** 911–917
28. Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., and Raetz, C. R. (1998) *J. Biol. Chem.* **273,** 12466–12475
29. Vorachek-Warren, M. K., Ramirez, S., Cotter, R. J., and Raetz, C. R. (2002) *J. Biol. Chem.* **277,** 14194–14205
30. Trent, M. S., Pabich, W., Raetz, C. R., and Miller, S. I. (2001) *J. Biol. Chem.* **276,** 9083–9092
31. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85
32. Garrett, T. A., Kadrmas, J. L., and Raetz, C. R. (1997) *J. Biol. Chem.* **272,** 21855–21864
33. Basu, S. S., York, J. D., and Raetz, C. R. (1999) *J. Biol. Chem.* **274,** 11139–11149
34. Belunis, C. J., and Raetz, C. R. (1992) *J. Biol. Chem.* **267,** 9988–9997
35. Guy-Caffey, J. K., Rapoza, M. P., Jolley, K. A., and Webster, R. E. (1992) *J. Bacteriol.* **174,** 2460–2465
36. Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972) *J. Biol. Chem.* **247,** 3962–3972
37. Dulbecco, R., and Vogt, M. (1954) *J. Exp. Med.* **99,** 167–182
38. Odegaard, T. J., Kaltashov, I. A., Cotter, R. J., Steeghs, L., van der Ley, P., Khan, S., Maskell, D. J., and Raetz, C. R. (1997) *J. Biol. Chem.* **272,** 19688–19696
39. Doerrler, W. T., and Raetz, C. R. (2002) *J. Biol. Chem.* **277,** 36697–36705
40. Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. (2000) *EMBO J.* **19,** 5071–5080
41. Brozek, K. A., Bulawa, C. E., and Raetz, C. R. (1987) *J. Biol. Chem.* **262,** 5170–5179
42. Belden, W. J., and Miller, S. I. (1994) *Infect. Immun.* **62,** 5095–5101
43. Gunn, J. S., Belden, W. J., and Miller, S. I. (1998) *Microb. Pathog.* **25,** 77–90
44. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) *Cell* **95,** 189–198
45. Icho, T. (1988) *J. Bacteriol.* **170,** 5117–5124
46. Karbarz, M. J., Kalb, S. R., Cotter, R. J., and Raetz, C. R. (2003) *J. Biol. Chem.* **278,** 39269–39279
47. Dillon, D. A., Wu, W. I., Riedel, B., Wissing, J. B., Dowhan, W., and Carman, G. M. (1996) *J. Biol. Chem.* **271,** 30548–30553
48. Icho, T., and Raetz, C. R. (1983) *J. Bacteriol.* **153,** 722–730
49. Wheeler, D. L., Church, D. M., Federhen, S., Lash, A. E., Madden, T. L., Pontius, J. U., Schuler, G. D., Schriml, L. M., Sequeira, E., Tatusova, T. A., and Wagner, L. (2003) *Nucleic Acids Res.* **31,** 28–33
50. Tatusov, R. L., Galperin, M. Y., Natale, D. A., and Koonin, E. V. (2000) *Nucleic Acids Res.* **28,** 33–36
51. Tatusov, R. L., Koonin, E. V., and Lipman, D. J. (1997) *Science* **278,** 631–637
52. Tatusov, R. L., Natale, D. A., Garkavtsev, I. V., Tatusova, T. A., Shankavaram, U. T., Rao, B. S., Kiryutin, B., Galperin, M. Y., Fedorova, N. D., and Koonin, E. V. (2001) *Nucleic Acids Res.* **29,** 22–28
53. Cox, A. D., Wright, J. C., Li, J., Hood, D. W., Moxon, E. R., and Richards, J. C. (2003) *J. Bacteriol.* **185,** 3270–3277
54. Trent, M. S., and Raetz, C. R. H. (2002) *J. Endotoxin Res.* **8,** 158
55. Wang, X., Karbarz, M. J., McGrath, S. C., Cotter, R. J., and Raetz, C. R. (2004) *J. Biol. Chem.* **279,** 49470–49478
56. Monteiro, M. A., Appelmelk, B. J., Rasko, D. A., Moran, A. P., Hynes, S. O., MacLean, L. L., Chan, K. H., Michael, F. S., Logan, S. M., O'Rourke, J., Lee, A., Taylor, D. E., and Perry, M. B. (2000) *Eur. J. Biochem.* **267,** 305–320
57. Moran, A. P., Prendergast, M. M., and Appelmelk, B. J. (1996) *FEMS Immunol. Med. Microbiol.* **16,** 105–115
58. Trent, M. S. (2004) *Biochem. Cell Biol.* **82,** 71–86
59. Que-Gewirth, N. L., Karbarz, M. J., Kalb, S. R., Cotter, R. J., and Raetz, C. R. (2003) *J. Biol. Chem.* **278,** 12120–12129
60. Que-Gewirth, N. L., Lin, S., Cotter, R. J., and Raetz, C. R. (2003) *J. Biol. Chem.* **278,** 12109–12119
61. Stukey, J., and Carman, G. M. (1997) *Protein Sci.* **6,** 469–472
62. Carman, G. M. (1997) *Biochim. Biophys. Acta* **1348,** 45–55
63. Johnson, A. G. (1994) *Clin. Microbiol. Rev.* **7,** 277–289
64. Johnson, A. G., Tomai, M., Solem, L., Beck, L., and Ribi, E. (1987) *Rev. Infect. Dis.* **9,** Suppl. 5, 512–516
65. Tomai, M. A., Solem, L. E., Johnson, A. G., and Ribi, E. (1987) *J. Biol. Response Modif.* **6,** 99–107
66. Chang, G., and Roth, C. B. (2001) *Science* **293,** 1793–1800
67. Doerrler, W. T., Reedy, M. C., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 11461–11464
68. Schmitt, L. (2002) *Chembiochem.* **3,** 161–165
69. Trent, M. S., Ribeiro, A. A., Doerrler, W. T., Lin, S., Cotter, R. J., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 43132–43144
70. Trent, M. S., Ribeiro, A. A., Lin, S., Cotter, R. J., and Raetz, C. R. (2001) *J. Biol. Chem.* **276,** 43122–43131
71. Doerrler, W. T., Gibbons, H. S., and Raetz, C. R. (2004) *J. Biol. Chem.* **279,** 45102–45109
72. Lee, H., Hsu, F. F., Turk, J., and Groisman, E. A. (2004) *J. Bacteriol.* **186,** 4124–4133
73. Zhou, Z., Ribeiro, A. A., and Raetz, C. R. (2000) *J. Biol. Chem.* **275,** 13542–13551
74. Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S. I. (1998) *Mol. Microbiol.* **27,** 1171–1182
75. Mitaku, S., Ono, M., Hirokawa, T., Boon-Chieng, S., and Sonoyama, M. (1999) *Biophys. Chem.* **82,** 165–171
76. Hirokawa, T., Boon-Chieng, S., and Mitaku, S. (1998) *Bioinformatics* **14,** 378–379
77. Wang, R. F., and Kushner, S. R. (1991) *Gene (Amst.)* **100,** 195–199