

The Lipid A 1-Phosphatase of *Helicobacter pylori* Is Required for Resistance to the Antimicrobial Peptide Polymyxin

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Modification of the phosphate groups of lipid A with amine-containing substituents, such as phosphoethanolamine, reduces the overall net negative charge of gram-negative bacterial lipopolysaccharide, thereby lowering its affinity to cationic antimicrobial peptides. Modification of the 1 position of *Helicobacter pylori* lipid A is a two-step process involving the removal of the 1-phosphate group by a lipid A phosphatase, LpxE_{HP} (Hp0021), followed by the addition of a phosphoethanolamine residue catalyzed by EptA_{HP} (Hp0022). To demonstrate the importance of modifying the 1 position of *H. pylori* lipid A, we generated LpxE_{HP}-deficient mutants in various *H. pylori* strains by insertion of a chloramphenicol resistance cassette into *lpxE_{HP}* and examined the significance of LpxE with respect to cationic antimicrobial peptide resistance. Using both mass spectrometry analysis and an in vitro assay system, we showed that the loss of LpxE_{HP} activity in various *H. pylori* strains resulted in the loss of modification of the 1 position of *H. pylori* lipid A, thus confirming the function of LpxE_{HP}. Due to its unique lipid A structure, *H. pylori* is highly resistant to the antimicrobial peptide polymyxin (MIC > 250 μg/ml). However, disruption of *lpxE_{HP}* in *H. pylori* results in a dramatic decrease in polymyxin resistance (MIC, 10 μg/ml). In conclusion, we have characterized the first gram-negative LpxE-deficient mutant and have shown the importance of modifying the 1 position of *H. pylori* lipid A for resistance to polymyxin.

Helicobacter pylori is a prevalent human pathogen infecting the stomachs of nearly half of the world's population (43). Although most *H. pylori* infections are asymptomatic, chronic infection of the gastric mucosa by the bacterium causes recurrent gastroduodenal inflammatory disease (4, 55). In addition, persistent infection with *H. pylori* is considered a risk factor for the development of mucosa-associated lymphoid tissue lymphoma, loss of gastric glands (atrophy), and adenocarcinoma (14, 41). A unique feature of *H. pylori* is its ability to persist in the human gastric mucosa, an ecological niche in which no other microorganism is known to thrive. *H. pylori* persistence in the mucosa is dependent on a number of different virulence factors, including production of vacuolating toxin, adhesions, urease, and presumably like other mucosal pathogens, the ability to resist the action of cationic antimicrobial peptides (CAMPs) (5, 7).

CAMPs are key components of the innate immune response and play an important role in the host defense against microbial infection (21, 31, 32). Although the exact mechanisms by which CAMPs kill gram-negative bacteria are not well understood, it has been proposed that the antimicrobial activity of CAMPs is initiated predominately through binding to the lipid A moiety of lipopolysaccharide (LPS) (9, 50). LPS is the major surface molecule of gram-negative bacteria and is held in the outer membrane by its hydrophobic anchor, lipid A (39, 45). The lipid A moiety of LPS is the bioactive component of LPS associated with endotoxic shock and is required to maintain

the integrity of the outer membrane barrier (45, 59). The electrostatic interaction between CAMPs and the negatively charged phosphate groups of lipid A disrupts the structure and integrity of the outer membrane, causing the membrane barrier to become more permeable. Permeabilization of the outer membrane allows CAMPs to gain access to the inner membrane, where they are thought to impose their bactericidal effects (62, 63).

Microorganisms have evolved several mechanisms to avoid being killed by CAMPs, including covalent modification of gram-negative bacterial lipid A (59). In some organisms, such as *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*, modification of the lipid A structure is under the control of the two-component regulatory systems PhoP/PhoQ and PmrA/PmrB (13, 15, 18). For example, activation of *Salmonella* PmrA results in the masking of the lipid A phosphate groups with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN). Addition of these amine-containing substituents results in reduction in the overall negative charge of the lipid A domain of LPS, promoting resistance to antimicrobial peptides, including polymyxin (15, 18). Polymyxin, a cyclic lipopeptide produced by the gram-positive soil bacterium *Paenibacillus polymyxa* (36), binds to the phosphate groups of lipid A and kills gram-negative bacteria in a manner that shares common features with the CAMPs of the innate immune system (62, 63).

Instead of masking the negatively charged phosphate groups with positively charged moieties, some pathogenic bacteria, like *Francisella tularensis* (65), *Porphyromonas gingivalis* (28), *Bacteroides fragilis* (68), and *H. pylori* (37, 53), take an alternative approach by expressing enzymes that remove the lipid A

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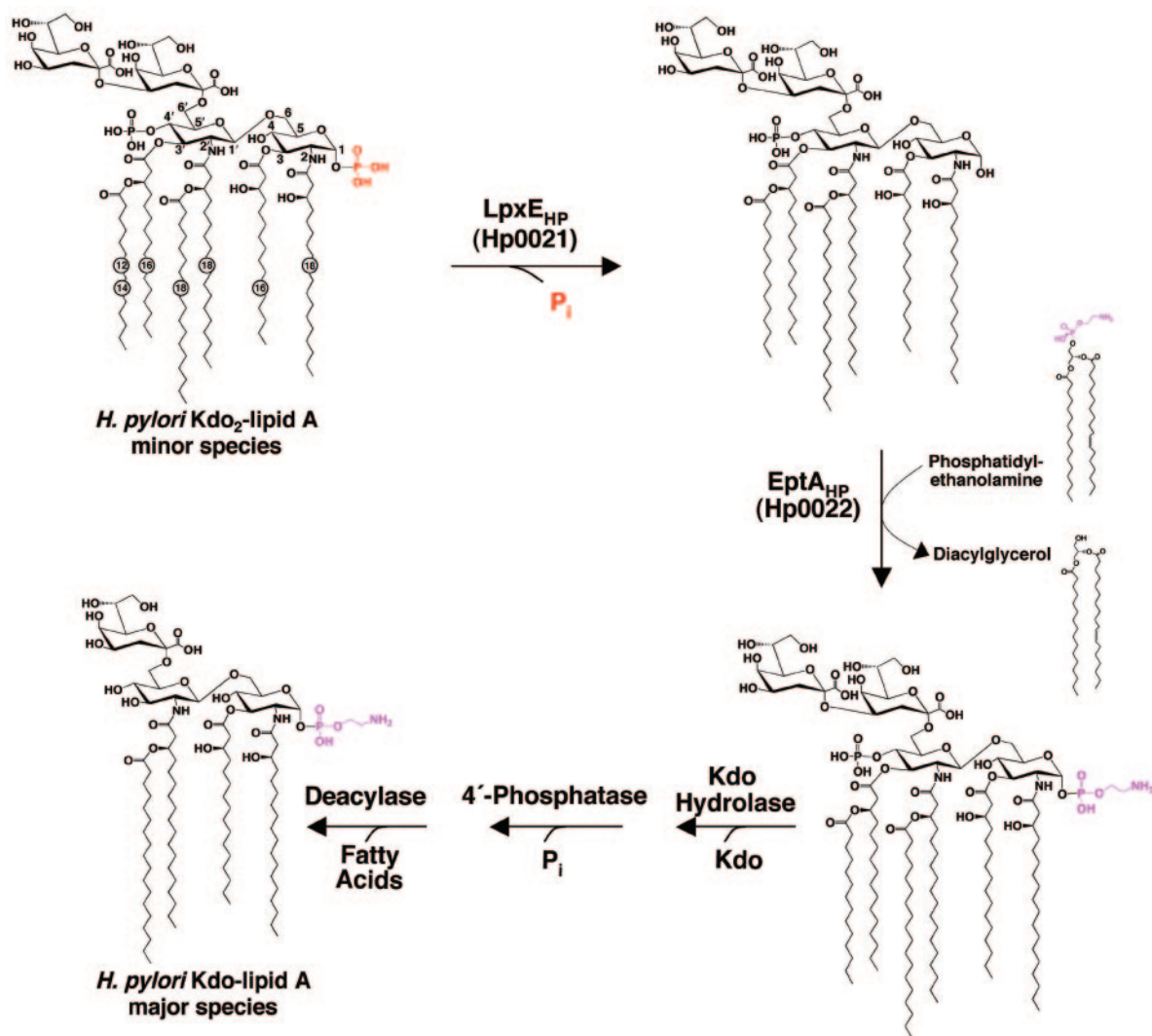


FIG. 1. The proposed reactions catalyzed by LpxE_{HP} (Hp0021) and EptA_{HP} (Hp0022) of *H. pylori*. *H. pylori* synthesizes a minor lipid A species that is bis-phosphorylated and hexa-acylated, resembling *E. coli* Kdo₂-lipid A (37, 58). Modification of the 1 position of *H. pylori* Kdo₂-lipid A is a two-step enzymatic process that involves the removal of the 1-phosphate group by LpxE_{HP}, followed by the addition of a pEtN unit directly to the glucosamine disaccharide backbone by EptA_{HP} (56). Further modifications to the *H. pylori* lipid A structure are catalyzed by a Kdo (3-deoxy-D-manno-octulosonic acid) hydrolase (51), a 4'-phosphatase (A. X. Tran and M. S. Trent, unpublished data), and a 3'-O-deacylase (C. M. Stead and M. S. Trent, unpublished data), resulting in the major lipid A species produced by the organism (58).

phosphate groups from their LPSs. The first lipid A phosphatase, LpxE, was identified in the nitrogen-fixing gram-negative endosymbiont *Rhizobium leguminosarum* (26). Recently, LpxE homologs in both *Francisella novicida* and *H. pylori* have been cloned and characterized (56, 67). LpxE is distantly related to *Escherichia coli* PgpB, a phosphatidylglycerol phosphatase, and is highly selective for the 1-phosphate group of lipid A (26, 56, 67).

In *H. pylori*, removal of the lipid A 1-phosphate group is followed by addition of a pEtN residue to the C-1 hydroxyl, forming a phosphodiester linkage (Fig. 1). The reaction is catalyzed by the *Helicobacter* protein Hp0022 (EptA_{HP}) and occurs only after removal of the 1-phosphate group (Fig. 1) (56). Hp0022 is a homolog of other described lipid A pEtN transferases, such as *E. coli* EptA (60), *S. enterica* serovar Typhimurium PmrC (30), and *Neisseria meningitidis* LptA (8).

However, these organisms do not express lipid A phosphatases and attach a pEtN residue directly to the lipid A phosphate group in a pyrophosphate linkage (30, 60). Previously, we demonstrated that membranes of *E. coli* containing LpxE_{HP} (Hp0021) catalyzed the dephosphorylation of lipid A (56). In this study, we generated the first gram-negative LpxE-deficient mutant and demonstrated that modification at the 1 position of *H. pylori* lipid A by LpxE plays an important role in *H. pylori* resistance to the antimicrobial peptide polymyxin.

MATERIALS AND METHODS

Chemicals and other materials. [γ -³²P]ATP and ³²P_i were obtained from Amersham International. Silica gel 60 (0.25-mm) thin-layer plates were purchased from EM Separation Technology (Merck). Yeast extract, tryptone, and brucella broth were from Difco. Triton X-100 and biconchonic acid were from Pierce. All other chemicals were reagent grade and were purchased from Sigma.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>Helicobacter pylori</i>		
J99	Wild type	ATCC 700824
26695	Wild type	ATCC 700392
Hp7-91	Clinical isolate	51
Cp20-84	Clinical isolate	51
Hsp110-93	Clinical isolate	51
26695/ <i>lpxE</i> _{HP} :: <i>cm</i>	26695 with chloramphenicol resistance cassette in Hp0021 gene	This work
J99/ <i>lpxE</i> _{HP} :: <i>cm</i>	J99 with chloramphenicol resistance cassette in Hp0021 gene	This work
Hp7-91/ <i>lpxE</i> _{HP} :: <i>cm</i>	<i>H. pylori</i> clinical isolate with chloramphenicol resistance cassette in Hp0021 gene	This work
Hp7-91/ <i>lpxE</i> _{HP} :: <i>cm</i> /pHel3- <i>lpxE</i> _{HP}	Hp7-91/ <i>lpxE</i> _{HP} :: <i>cm</i> ; pHel3 carrying Hp0021 gene	This work
Cp20-84/ <i>lpxE</i> _{HP} :: <i>cm</i>	<i>H. pylori</i> clinical isolate with chloramphenicol resistance cassette in Hp0021 gene	This work
Hsp110-93/ <i>lpxE</i> _{HP} :: <i>cm</i>	<i>H. pylori</i> clinical isolate with chloramphenicol resistance cassette in Hp0021 gene	This work
<i>Escherichia coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI</i> ^q Δ M15::Tn10 (Tet ^r)]	Stratagene
Plasmids		
pBluescript II SK(+)	Cloning vector; Amp ^r ; <i>lac</i> promoter (<i>lacZ</i>) f1 ColE1	Stratagene
pBShp0021	pBluescript II SK(+) containing Hp0021 gene	This work
pBShp0021NX	pBShp0021 engineered with NdeI and XhoI sites	This work
pBShp0021:: <i>cm</i>	pBShp0021NX containing a chloramphenicol resistance cassette in Hp0021 gene	This work
pHel2	<i>E. coli-H. pylori</i> shuttle vector; Cam ^r	23
pHel3	<i>E. coli-H. pylori</i> shuttle vector; Kan ^r	23
pHel3- <i>lpxE</i> _{HP}	pHel3 containing Hp0021 gene	This work

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are summarized in Table 1. *H. pylori* strains 26695 and J99 were obtained from the American Type Culture Collection. Clinical isolates of *H. pylori* were obtained from human gastric biopsy specimens, as previously described (33). *H. pylori* was cultivated in brucella broth supplemented with 10% fetal bovine serum (HyClone) and vancomycin (10 μ g/ml) or on blood agar plates (BAP) supplemented with vancomycin (10 μ g/ml), chloramphenicol (8 μ g/ml), and/or kanamycin (8 μ g/ml) when appropriate. The cells were grown under microaerobic conditions as previously described (56). *E. coli* strain XL-1 Blue was routinely grown in LB broth or on LB agar (46) supplemented with ampicillin (100 μ g/ml), chloramphenicol (30 μ g/ml), and/or kanamycin (30 μ g/ml) when appropriate.

Recombinant DNA techniques. Plasmids were isolated using the QIAGEN Spin Prep kit. Custom primers were obtained from Integrated DNA Technologies. PCR reagents were purchased from Stratagene. PCR cleanup was performed using the QIAquick PCR Purification kit (QIAGEN). DNA fragments were isolated from agarose 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.

Natural transformation of *H. pylori*. Natural transformation of *H. pylori* strains was performed with plasmid DNA according to the procedures described by Haas et al. (19). In brief, bacteria were harvested from BAP and suspended to an A_{600} of 0.1 in brucella broth medium containing 10% fetal bovine serum and vancomycin (10 μ g/ml). The cells were incubated for 6 to 12 h under microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂) at 37°C to allow nonmotile cells to recover. Afterward, plasmid DNA (2.5 to 5 μ g) was added, and the cells were incubated for another 6 h before the suspension was plated on selective BAP. Resistant colonies were repurified on chloramphenicol (8 μ g/ml)- and/or kanamycin (8 μ g/ml)-containing plates.

Construction and complementation of an *H. pylori lpxE*_{HP} (Hp0021 gene)-deficient mutant. The Hp0021 gene and its flanking sequences, including 654 base pairs upstream and 807 base pairs downstream, were amplified by PCR (primers U-*hp0021* and L-*hp0021*) (Table 2) from *H. pylori* 26695 genomic DNA using *Pfu* Turbo (Stratagene) according to the manufacturer's instructions. The flanking DNA was digested with BamHI and KpnI, gel purified, and subsequently cloned into pBluescript II SK(+) (Stratagene), resulting in the plasmid pBShp0021. The vector pBShp0021 was then subjected to two separate rounds of site-directed mutagenesis, using the QuikChange XL Site-Directed Mutagenesis kit (Stratagene), to create both NdeI (primers U-*hp0021*NdeI and L-*hp0021*NdeI) and XhoI (primers U-*hp0021*XhoI and L-*hp0021*XhoI) restriction sites (pBShp0021NX). In order to disrupt the Hp0021 gene, a chloram-

phenicol resistance cassette (*cm*), obtained by PCR (primers U-*hp0021*CAM and L-*hp0021*CAM) from the *E. coli-H. pylori* shuttle vector (pHel2) (23), was inserted into the NdeI and XhoI sites of pBShp0021NX. The resulting suicide plasmid, pBShp0021::*cm*, containing an interrupted Hp0021 gene, was transformed into the *H. pylori* wild-type strains shown in Table 1 by natural transformation (23), and resistant colonies were selected on BAP containing 8 μ g/ml of chloramphenicol. Resistant colonies were repurified on chloramphenicol-containing plates, and the successful insertion of the resistance cassette was verified by PCR of genomic DNA to ensure a double crossover (data not shown). Additionally, PCR primers were designed for amplification of the constructed pBluescript II SK(+) suicide vector to confirm that no vector DNA remained in the genome (data not shown).

To construct a plasmid for complementation of the *lpxE* mutant, the *lpxE* gene, including 150 base pairs upstream, was amplified by PCR (U-*hp0021*Comp and L-*hp0021*Comp) from *H. pylori* Hp7-91 genomic DNA using *Pfu* Turbo (Stratagene) according to the manufacturer's instructions. The PCR product was digested with XhoI and KpnI, gel purified, and cloned into the *E. coli-H. pylori* shuttle vector (pHel3) (23). The resulting plasmid, pHel3-*lpxE*_{HP}, was transformed into *H. pylori lpxE*_{HP}-deficient mutants by natural transformation and selected on BAP containing chloramphenicol (8 μ g/ml) and kanamycin (8 μ g/ml).

Isolation and analysis of lipid A and phospholipid species from ³²P_i-labeled

TABLE 2. Oligonucleotides

Name	Sequence ^a
U- <i>hp0021</i>	5'-GCGCGCGGATCCAACGACGGCGAAAAGAAT-3'
L- <i>hp0021</i>	5'-GCGCGCGGTACCAGGATAAACCTCTCTAT-3'
U- <i>hp0021</i> NdeI	5'-GTTTGGGCGGAGCGCCCATATGGAGGTAATTC AAC-3'
L- <i>hp0021</i> NdeI	5'-GTTGAAATTACCTCCATATGGGGCTCGCCCA AAC-3'
U- <i>hp0021</i> XhoI	5'-AACATGCCAAGCGGGCACTCGAGTATGGTGG GTTTGGCGGTG-3'
L- <i>hp0021</i> XhoI	5'-CACCGCCAAACCCACCATACTCGAGTGCCCGC TTGGCATGTT-3'
U- <i>hp0021</i> CAM	5'-GCGCGCCATATGCCGAGATTTTCAGGAGCT-3'
L- <i>hp0021</i> CAM	5'-GCGCGCCTCGAGTTACGCCCGCTGCCA-3'
U- <i>hp0021</i> Comp	5'-GCGCGCCTCGAGAGCATCCATCATCAA-3'
L- <i>hp0021</i> Comp	5'-GCGCGCGGTACCTTAAGGTTTTTGATTA-3'

^a Underlining indicates restriction sites.

cells. *H. pylori* strains were grown as described above. Typically, a 20-ml cell culture was labeled uniformly with 5 $\mu\text{Ci/ml}$ $^{32}\text{P}_i$ until the cells reached late log phase. Bacteria were collected using a clinical centrifuge and washed with 5 ml of phosphate-buffered saline (pH 7.4). ^{32}P -labeled lipid A and phospholipids were isolated using published protocols (70) and spotted onto a Silica Gel 60 thin-layer chromatography (TLC) plate ($\sim 10,000$ cpm/lane). Lipids were separated using the solvent chloroform, pyridine, 88% formic acid, and water (50:50:16:5 [vol/vol/vol/vol]). The TLC plates were exposed overnight to a phosphor-imager screen, and product formation was detected and analyzed using a Bio-Rad Molecular Imager phosphorimager equipped with Quantity One software.

Large-scale isolation of *H. pylori* lipid A. Cultures (500 ml) were grown to an A_{600} of 1.0 at 37°C under microaerophilic conditions for 36 to 40 h. Cells were harvested by centrifugation at $6,000 \times g$ for 15 min, washed once with phosphate-buffered saline, and resuspended in 20 ml of phosphate-buffered saline (12). Lipid A was released from the cells and separated by DEAE anion-exchange chromatography as previously described (40, 70) and stored frozen at -20°C .

Mass spectrometry of lipid A species. Mass spectra of the purified lipids were acquired in the negative linear mode using a matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF) mass spectrometer (AXIMA-CFR; Kratos Analytical, Manchester, United Kingdom) 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 unit for compounds with an M_r of $\sim 2,000$. Each spectrum represented the average of 100 laser shots. Saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1 [vol/vol]) served as the matrix. The samples were dissolved in chloroform/methanol (4:1 [vol/vol]) 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.

Preparation of cell extracts, double-spun cytosol, and washed membrane. Typically, 250 ml of *H. pylori* cultures was grown to an A_{600} of 1.0 at 37°C and harvested by centrifugation at $6,000 \times g$ for 30 min. All samples were prepared at 4°C. Cell extract, membrane-free cytosol, and washed membranes were prepared as previously described (56) and stored in aliquots at -20°C . The protein concentration was determined by the bicinchoninic acid method (49), using bovine serum albumin as the standard.

Preparation of radiolabeled substrates. The substrate [$4'$ - ^{32}P]lipid IV_A was generated from 125 μCi of [γ - ^{32}P]ATP and the tetra-acyl-disaccharide 1-phosphate lipid acceptor (a generous gift from C. R. H. Raetz), using the overexpressed 4'-kinase (LpxK) present in membranes of *E. coli* BLR(DE3)/pLysS/pJK2, as previously described (1, 2, 56).

***H. pylori* lipid A 1-phosphatase assay.** The *H. pylori* lipid A 1-phosphatase activity was assayed under optimized conditions based on the method of Tran et al. (56). Reaction mixtures (10 μl) contained 50 mM MES (morpholineethanesulfonic acid) (pH 6.0), 0.2% Triton X-100, and 5 μM [$4'$ - ^{32}P]lipid IV_A ($\sim 3,000$ to 5,000 cpm/nmol) as the substrate. Washed membranes (1.0 mg/ml) were employed as the enzyme source, as indicated. Dephosphorylation reaction mixtures were incubated at 30°C for the indicated times and terminated by spotting 4.5- μl portions of the mixtures onto silica gel 60 TLC plates. Reaction products were separated and visualized as described above.

Polymyxin B sensitivity assays. Polymyxin B sulfate (Sigma) was used at concentrations of 0.1 to 250 $\mu\text{g/ml}$ in both plate and broth assays. Standard MIC testing of susceptibility to polymyxin was performed as described by Gunn and Miller and Steinberg et al. (16, 52). To assay *H. pylori* strains for polymyxin resistance, the strains were grown to mid-log phase (optical density at 600 nm, ~ 0.35) and diluted to a concentration of approximately 2,500 CFU/ml in brucella broth. The cells (200 μl) were mixed on a microtiter plate with various concentrations of polymyxin B sulfate (Sigma) and incubated at 37°C for 1 h. Then, 150 μl of polymyxin-treated cells was directly plated on BAP and incubated at 37°C under microaerobic conditions for 36 to 40 h. The colony counts of cells incubated with the various concentrations of polymyxin were compared to colony counts of cells not exposed to polymyxin. The percent survival was defined as follows: survival (%) = (CFU of peptide-exposed culture/CFU of nonexposed culture) \times 100.

Transmission electron microscopy. *H. pylori* cells were grown to mid-log phase (A_{600} , ~ 0.5) and diluted to a concentration of approximately 2,500 CFU/ml in brucella broth. Cells (2 ml) were exposed to 100 $\mu\text{g/ml}$ of polymyxin B sulfate (Sigma) and incubated at 37°C under microaerobic conditions for 1 h. Afterward, samples were washed, fixed in 2% (vol/vol) glutaraldehyde plus 0.5% (vol/vol) paraformaldehyde, processed, embedded in Epon-Araldite resin, and stained for high-contrast morphology as described previously (25, 69). Ultrathin sections were examined with a Philips Tecnai 10 transmission electron microscope (FEI Company, Hillsboro, Ore.) operating at 80 kV.

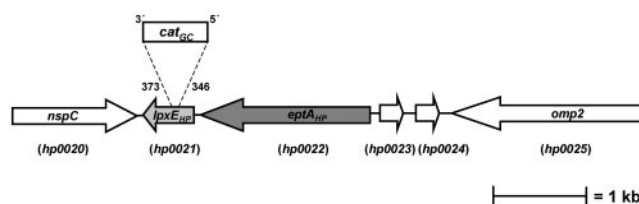


FIG. 2. Schematic representation of the *H. pylori* chromosomal region of strain 26695 encoding LpxE_{HP} and EptA_{HP}. The overall genomic organizations of *H. pylori* genes encoding enzymes modifying the 1 position of *H. pylori* lipid A are similar in strains 26695 and J99. However, the region between the Hp0022 and Hp0025 genes are not annotated as open reading frames in strain J99. As indicated in the figure, 28 bp of DNA was removed from the *lpxE*_{HP} gene prior to insertion of the *catGC* cassette conferring chloramphenicol resistance. Based upon homology to other lipid phosphatases, such as *Francisella* LpxE, *Rhizobium* LpxE, and *E. coli* PgpB, the deleted 28-bp region encodes a protein domain that is predicted to function as part of the active site of *H. pylori* LpxE.

RESULTS

MALDI-TOF mass spectrometry and ^{32}P labeling of the lipids A from *H. pylori* strains 26695 and 26695/*lpxE*_{HP}::*cm*. In strain 26695, the Hp0021 (accession number DQ447324) and Hp0022 (accession number DQ447325) genes encode the lipid A 1-phosphatase and pEtN transferase, respectively (56) (Fig. 2), with a similar genomic organization found in strain J99. In either case, these genes are unidirectionally transcribed beginning with the Hp0022 gene and separated by only 10 bp. To determine if modification of *H. pylori* lipid A at the 1 position was important for resistance to antimicrobial peptides and to confirm its role in the modification of *Helicobacter* lipid A, we constructed a *lpxE*_{HP}-deficient mutant of the sequenced strain 26695. Based upon previously reported biochemical evidence (56), it was expected that inactivation of *lpxE*_{HP} would result in the loss of 1-phosphatase activity, thereby preventing the addition of pEtN catalyzed by *H. pylori* EptA_{HP} (Hp0022).

The lipid A species from both wild-type 26695 and the 26695/*lpxE*_{HP}::*cm* mutant were isolated and fractionated based upon their charges by DEAE-cellulose column chromatography with increasing salt step elutions. Following DEAE purification, the lipids were analyzed by MALDI-TOF mass spectrometry in the negative mode. Mass spectrometry analysis of wild-type 26695 showed a major ion peak at m/z 1549.0 atomic mass units, corresponding to a tetra-acylated lipid A structure that lacks the 4'-phosphate group and is substituted at the C-1 position with a pEtN residue (lipid I) (Fig. 3A). This is the first analysis of the lipid A structure synthesized by one of the sequenced strains of *H. pylori* and is identical to that previously reported by Moran and coworkers for *H. pylori* strain NCTC 11637 (37). As predicted, inactivation of *lpxE*_{HP} in *H. pylori* strain 26695 prevented the enzymatic activity of EptA_{HP} and resulted in the presence of a tetra-acylated lipid A species with the 1-phosphate group still attached to the C-1 position (lipid II), as indicated by the major ion peak at m/z 1506.0 (Fig. 3B). Lipid II, bearing a single phosphate group, eluted from the DEAE column during the 120 mM salt elution. However, based upon the dry mass of lipid A fractions produced by the 26695 *lpxE* mutant, lipid II was $<5\%$ of the total lipid (data not shown).

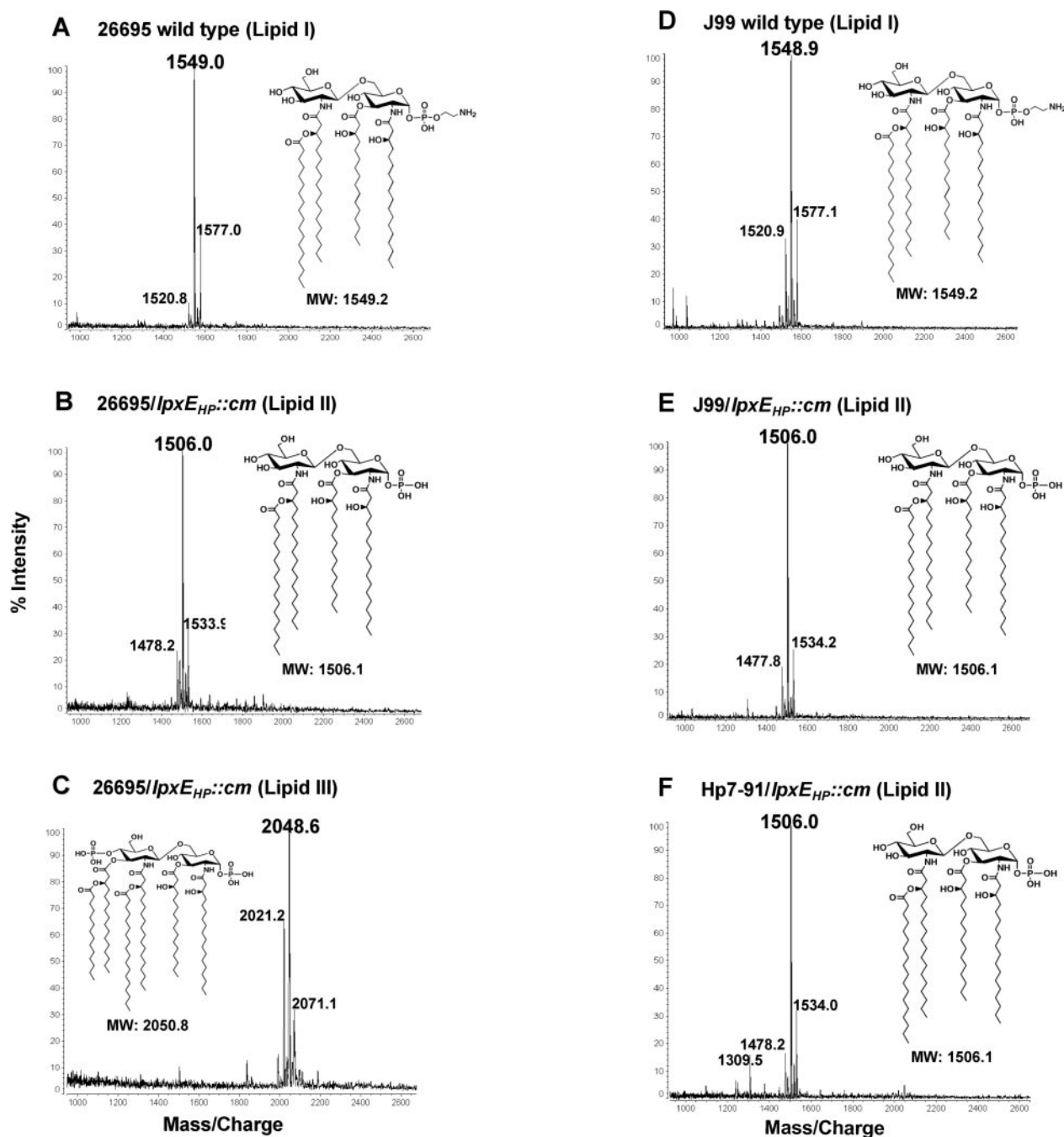


FIG. 3. Comparison of the lipid A fractions isolated from wild-type *H. pylori* and *lpxE_{HP}* mutants. Lipids A of the indicated strains were analyzed by MALDI-TOF mass spectrometry in the negative-ion mode. Although mass spectrometry data was acquired on lipids eluting in each fraction from the DEAE column (40), only those fractions containing lipids are shown. Lipid I from wild-type strains 26695 (A) and J99 (D) were found to elute in the flowthrough and wash fractions, whereas lipid II (B, E, and F) produced by *lpxE_{HP}* mutants eluted in the 120 mM salt fraction (see Materials and Methods). Lipid III (C), produced only by the 26695/*lpxE_{HP}*::*cm* mutant, eluted in the 240 mM salt fraction.

The bulk of the lipid A synthesized by 26695/*lpxE_{HP}*::*cm* eluted in the 240 mM salt fraction during DEAE chromatography, showing a major ion peak at m/z 2048.6 during mass spectrometry. The ion peak at m/z 2048.6 atomic mass units corresponds to a lipid A species retaining both the 1- and 4'-phosphate groups (lipid III), resulting in an increase in the negative charge. Furthermore, lipid III retained the 3'-ester-

linked fatty acyl chains, resulting in a hexa-acylated structure (Fig. 3C). TLC analysis of ^{32}P -labeled lipid A species isolated from 26695 and its isogenic *lpxE_{HP}* mutant confirmed that 26695/*lpxE_{HP}*::*cm* produces only one major lipid A species (Fig. 4A, lane 2) that migrates faster than wild-type lipid A (Fig. 4A, lane 1). The *bis*-phosphorylated and hexa-acylated lipid A structure synthesized by 26695/*lpxE_{HP}*::*cm*, lipid III,

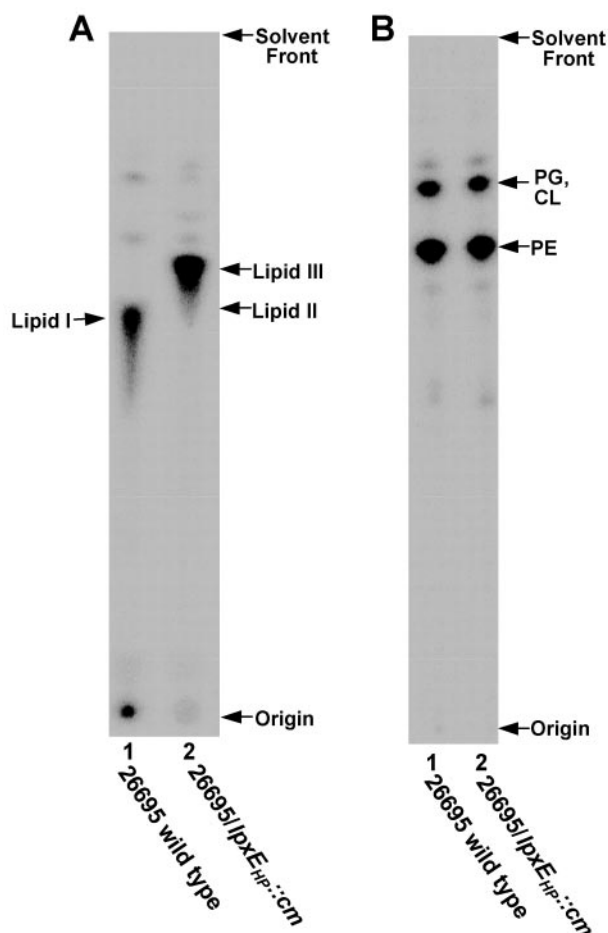


FIG. 4. Comparison of ^{32}P -labeled lipid A (A) and phospholipid fractions (B) isolated from *H. pylori* 26695 and 26695/*lpxE*_{HP}::*cm*. ^{32}P -labeled lipid A species or phospholipids were isolated as described in Materials and Methods and separated by TLC using the solvent chloroform, pyridine, 88% formic acid, and water (50:50:16:5 [vol/vol/vol/vol]). The ^{32}P -labeled lipids were visualized by phosphorimaging. The identities of the major phospholipids are based upon the major phospholipids reported for *H. pylori* (24) and the migration of the major phospholipids found in *E. coli* (72). PG, phosphatidylglycerol; PE, phosphatidylethanolamine; CL, cardiolipin.

confirms that *H. pylori* contains the necessary enzymatic machinery to synthesize a lipid A molecule structurally similar to that of *E. coli*. However, unlike *E. coli* lipid A, the lipid A of *Helicobacter* bears fatty acyl chains 16 to 18 carbons in length. Previously, Moran and coworkers and Tran et al. reported that a lipid A species identical to lipid III (Fig. 1) could be isolated from wild-type *H. pylori* strain NCTC 11637, but only as a minor component of the total lipid (37, 58). However, we were unable to detect any species similar to lipid III in wild-type strains grown in liquid culture. Given that LpxE is distantly related to *E. coli* PgpB, a phosphatidylglycerol phosphatase (11), the ^{32}P -labeled phospholipids of wild-type strain 26695 were compared to those of 26695/*lpxE*_{HP}::*cm* (Fig. 4B). Based upon TLC analysis, inactivation of *lpxE*_{HP} had no effect on phospholipid biosynthesis.

MALDI-TOF mass spectrometry of lipid A From *H. pylori* strains J99, J99/*lpxE*_{HP}::*cm*, and Hp7-91/*lpxE*_{HP}::*cm*. The *bis*-

phosphorylated and hexa-acylated lipid A structure resulting from disruption of *lpxE*_{HP} in 26695 (Fig. 3C) suggested that loss of 1-phosphatase activity may prevent other enzymatic modifications of *H. pylori* lipid A, such as removal of the 4'-phosphate or deacylation (Fig. 1) (58). Both pEtN addition and removal of the Kdo hydrolase have been shown to require prior removal of the 1-phosphate group in *H. pylori* (51, 56). Therefore, we were interested to determine whether inactivation of *lpxE*_{HP} in strain J99, a second strain for which the genomic sequence is available, resulted in production of similar lipid A structures. Like 26695, the outer membrane of *H. pylori* J99 contained a tetra-acylated lipid A lacking the 4'-phosphate group and bearing a pEtN at the 1 position (Fig. 3D). Mass spectrometry analysis of lipid A isolated from J99/*lpxE*_{HP}::*cm* revealed one major ion peak at *m/z* 1506.0 atomic mass units (Fig. 3E), resembling the minor tetra-acylated lipid A species (lipid II) of the 26695 *lpxE*_{HP} mutant. Interestingly, the tetra-acylated species bearing a single phosphate group was the major lipid produced by the J99 mutant, and no hexa-acylated structures were detected (Fig. 4).

Further investigation of three clinical isolates, Hp7-91 (Fig. 3F), Cp20-84, and Hsp110-93 (data not shown), lacking a functional 1-phosphatase, revealed that, like J99, these strains synthesized only a tetra-acylated molecule. Taken together, these data suggest that insertional inactivation of *lpxE*_{HP} results in replacement of a pEtN residue with a phosphate group at the C-1 position of *H. pylori* lipid A, increasing the overall negative charge of the molecule and, thus, of the bacterial surface. At present, it is unclear why the 26695 *lpxE*_{HP} mutant produces a lipid A species that is *bis*-phosphorylated and hexa-acylated.

Complementation of the *H. pylori* *lpxE*_{HP}-deficient mutant. Unlike strains 26695 and J99 (48), *H. pylori* Hp7-91 was transformed readily with the pHel3-*lpxE*_{HP} covering plasmid and therefore was selected for complementation studies. To confirm the enzymatic function of LpxE_{HP}, membranes of wild-type *H. pylori* strain Hp7-91 and Hp7-91/*lpxE*_{HP}::*cm* were isolated and assayed with [4'- ^{32}P]lipid IV_A, a key lipid A precursor, for 1-phosphatase activity. 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, denoted 1-dephospho-[4'- ^{32}P]lipid IV_A, when analyzed by TLC in the solvent system employed (56). As shown in Fig. 5A, membranes from wild-type *H. pylori* Hp7-91 displayed 1-phosphatase activity (Fig. 5A, lane 2). In contrast, membranes obtained from Hp7-91/*lpxE*_{HP} were essentially unable to catalyze the removal of the 1-phosphate group from [4'- ^{32}P]lipid IV_A (Fig. 5A, lane 3). To demonstrate that recovery of the 1-phosphatase activity was solely dependent upon LpxE, the *lpxE*_{HP} gene, along with 150 base pairs of DNA upstream of its start codon, was cloned into the *E. coli*/*H. pylori* shuttle vector, pHel3 (23). The resulting plasmid, pHel3-*lpxE*_{HP}, was used to complement the *lpxE*_{HP} mutant, resulting in restoration of 1-phosphatase activity (Fig. 5, lane 4). Introduction of the vector control, pHel3, into Hp7-91/*lpxE*_{HP}::*cm* had no effect upon 1-phosphatase activity (data not shown). Furthermore, natural transformation of pHel3-*lpxE*_{HP} into Hp7-91/*lpxE*_{HP}::*cm* restored the synthesis of a lipid A structure identical to that of wild-type *H. pylori* showing a predominant peak at *m/z* 1548.1 atomic mass units during mass spectrometry (Fig. 5B).

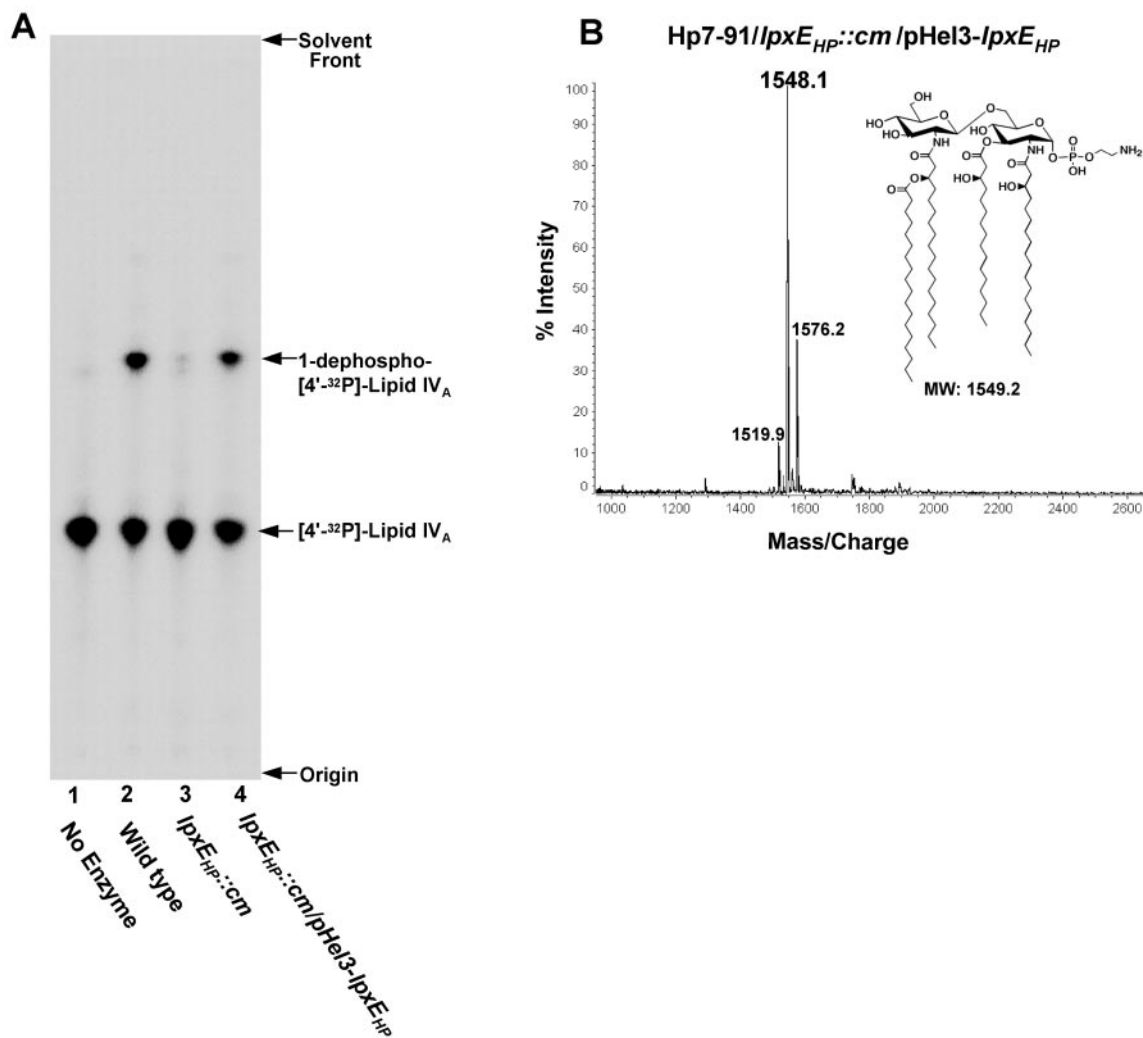


FIG. 5. Reconstitution of 1-phosphatase activity in membranes of an *H. pylori* Hp7-91/*lpxE_{HP}* mutant by the introduction of an *lpxE_{HP}*-complementing plasmid (A) and MALDI-TOF mass spectrometry of lipid A obtained from the complemented Hp7-91/*lpxE_{HP}*::*cm* mutant (B). Membranes isolated from the indicated *H. pylori* strains were assayed for 1-phosphatase activity under conditions described in Materials and Methods. Protein concentrations were 1.0 mg/ml, and the assays were carried out for 1 h at 30°C. Each reaction mixture contained 5 μM of [4'-³²P]lipid IV_A substrate. The reaction products were separated by TLC and detected by phosphorimager analysis. Removal of the 1-phosphate group was indicated by conversion of the [4'-³²P]lipid IV_A substrate to a faster-migrating species (56). (B) The lipid A of Hp7-91/*lpxE_{HP}*::*cm* transformed with pHel3-*lpxE_{HP}* was isolated and fractionated by DEAE-cellulose chromatography using published protocols (40). The lipid A sample was analyzed by MALDI-TOF mass spectrometry in the negative-ion mode. The data shown are from lipid A species found to elute in the flowthrough and wash fractions.

Disruption of *lpxE_{HP}* affects *H. pylori* resistance to polymyxin. Polymyxin primarily interacts with the negatively charged phosphate groups of lipid A. *H. pylori* is intrinsically resistant to polymyxin (Table 3), presumably due in part to the low number of negative charges in its lipid A. Modification of

H. pylori lipid A, including pEtN decoration of the C-1 hydroxyl group, does not appear to require additional signals when the bacterium is cultured under standard laboratory conditions. The direct attachment of pEtN to the 1 position of the disaccharide backbone of *Helicobacter* lipid A results in a greater reduction in the negative charge than the pyrophosphate linkage from pEtN addition in *E. coli* (70), *S. enterica* serovar Typhimurium (71), and *N. meningitidis* (27).

To demonstrate the importance of modifying the 1 position of *H. pylori* lipid A and to examine the significance of LpxE_{HP} for polymyxin resistance, wild-type *H. pylori* and *lpxE_{HP}* mutants were exposed to increasing concentrations of polymyxin and examined for the ability to survive in comparison to bacteria not exposed to the peptide (Fig. 6). As shown in Fig. 6, wild-type *H. pylori* strain 26695 demonstrated nearly 100% survival even at a polymyxin concentration of 100 μg/ml. Ad-

TABLE 3. MICs of polymyxin of *H. pylori* strains

Strain	Polymyxin MIC (μg/ml)
26695	>250
26695/ <i>lpxE_{HP}</i> :: <i>cm</i>	0.2
J99	>250
J99/ <i>lpxE_{HP}</i> :: <i>cm</i>	10
Hp7-91	>250
Hp7-91/ <i>lpxE_{HP}</i> :: <i>cm</i>	25
Hp7-91/ <i>lpxE_{HP}</i> :: <i>cm</i> /pHel3- <i>lpxE_{HP}</i>	>250

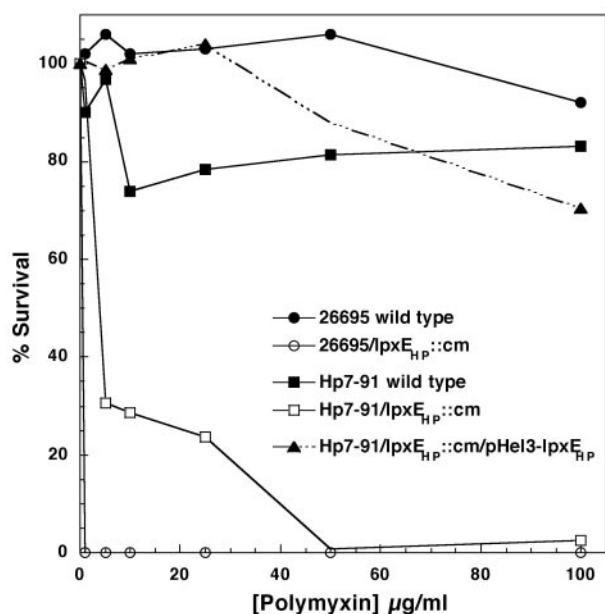


FIG. 6. Effect of *lpxE_{HP}* mutation on the polymyxin resistance of *H. pylori*. *H. pylori* strains were exposed to increasing concentrations of polymyxin, and the survival percentages were determined as described in Materials and Methods. Wild-type strains 26695 and Hp7-91 served as the polymyxin-resistant controls. Survival data for strain J99 were similar to those shown for strain Hp7-91 (data not shown).

ditionally, we determined the MICs of polymyxin for all wild-type strains to be greater than 250 µg/ml (Table 3).

Disruption of *lpxE_{HP}* in all strains resulted in a dramatic decrease in the percent survival when exposed to polymyxin (Fig. 6 and Table 3). The MICs of 26695/*lpxE_{HP}*::*cm* and J99/*lpxE_{HP}*::*cm* were found to be 0.2 and 10 µg/ml, respectively (Table 3). The differences in polymyxin sensitivity between the two *lpxE_{HP}* mutant strains can be attributed to the number of phosphate groups on the lipid A molecule that are exposed to attack by polymyxin. The lipid A isolated from 26695/*lpxE_{HP}*::*cm* resembles the *bis*-phosphorylated lipid A structure of unmodified *E. coli* lipid A (45), which is also highly sensitive to polymyxin with a MIC of 1 µg/ml (64). Having two phosphates attached to the lipid A domain of 26695/*lpxE_{HP}*::*cm* dramatically increased the sensitivity to polymyxin by more than 1,000-fold compared to wild-type 26695 (Table 3). However, the sensitivity to polymyxin of strain J99/*lpxE_{HP}*::*cm*, which has a single phosphate group attached to lipid A at the C-1 position, increased by 25-fold compared to wild-type J99 (Table 3). As shown in Table 3, 26695/*lpxE_{HP}*::*cm* was 50 times more sensitive to polymyxin than J99/*lpxE_{HP}*::*cm*, demonstrating the importance of modifying the lipid A phosphate groups of *H. pylori* LPS for resistance to CAMPs in *H. pylori*.

The level of polymyxin resistance was also investigated in *H. pylori* strains Hp7-91 and Hp7-91/*lpxE_{HP}*::*cm* and the complemented Hp7-91 mutant. The percent survival and MIC of polymyxin for the *H. pylori* clinical isolate Hp7-91 were similar to those observed for wild-type 26695 and J99 (Fig. 6 and Table 3). The lipid A structures of both Hp7-91/*lpxE_{HP}*::*cm* and J99/*lpxE_{HP}*::*cm* were identical when analyzed by mass spec-

trometry and therefore displayed similar sensitivities to polymyxin (Table 3). Polymyxin resistance was fully restored in Hp7-91/*lpxE_{HP}*::*cm* upon introduction of the *lpxE_{HP}* covering plasmid (Fig. 6 and Table 3), ruling out any possible polar effects.

Transmission electron microscopy of the *H. pylori* 1-phosphatase mutant following exposure to polymyxin. Having demonstrated that deletion of *lpxE_{HP}* in *H. pylori* dramatically reduces resistance to polymyxin, we next examined the effect of polymyxin on the bacterial morphology of the *lpxE* mutant by transmission electron microscopy. Electron micrographs of both wild-type Hp7-91 and Hp7-91/*lpxE_{HP}*::*cm* that were not exposed to polymyxin showed rods or curved rods (data not shown). Exposure to polymyxin at 100 µg/ml did not affect the cell morphology of wild-type Hp7-91 (Fig. 7A). Conversely, exposure of bacteria to polymyxin lacking a functional LpxE_{HP} resulted in morphological alterations resembling those reported for *H. pylori* undergoing coccoid formation, eventually leading to cell death (Fig. 7B to D) (3, 29). The cell-damaging effect of polymyxin was characterized by ruffling of the outer membrane, membrane invaginations, and in some cases an overall increase in the periplasmic region of the bacterial cell envelope (Fig. 7B to D). Restoration of polymyxin resistance in Hp7-91/*lpxE_{HP}*::*cm* was observed upon transformation of the plasmid pHel3-*lpxE_{HP}* (Fig. 7E and F).

DISCUSSION

H. pylori heavily modifies the lipid A moiety of its LPS, resulting in the production of a structure with a hydroxyl group at the 4' position and a pEtN residue at the 1 position (Fig. 1) (58). The unique structure of *H. pylori* lipid A should help promote resistance to CAMPs during host infection, since it is well known that the lipid A phosphate groups are necessary for binding of cationic peptides (59). Here, we report the characterization of an *H. pylori* mutant unable to express functional LpxE_{HP}, a lipid A 1-phosphatase. Insertional inactivation of *lpxE_{HP}* resulted in the loss of 1-phosphatase activity and the subsequent transfer of pEtN. These findings corroborated our previous report that the *H. pylori* lipid A pEtN transferase was unique in that it functions only after the removal of the 1-phosphate group by LpxE_{HP} (56).

Wild-type *H. pylori* strains were found to be inherently resistant to the antimicrobial peptide polymyxin, displaying a MIC of >250 µg/ml. This high level of polymyxin resistance has also been reported for other gram-negative organisms, including *Proteus mirabilis* (35), *Brucella abortus* (34), and *N. meningitidis* (61). *H. pylori* strain J99 and three additional clinical isolates lacking a functional LpxE_{HP} all synthesized lipid A structures bearing a single phosphate group at the 1 position, resulting in up to a 25-fold increase in polymyxin sensitivity. Introduction of a complementing plasmid expressing the phosphatase completely restored polymyxin resistance (Fig. 6B), correlating with a lipid A structure bearing a pEtN group at the C-1 position (Fig. 5B). An unexpected but reproducible finding of these studies was the synthesis of a *bis*-phosphorylated species in the 26695 *lpxE_{HP}* mutant. Interestingly, the 26695 *lpxE_{HP}* mutant displayed complete sensitivity to polymyxin (MIC, 0.2 µg/ml) in comparison to other strains, suggesting

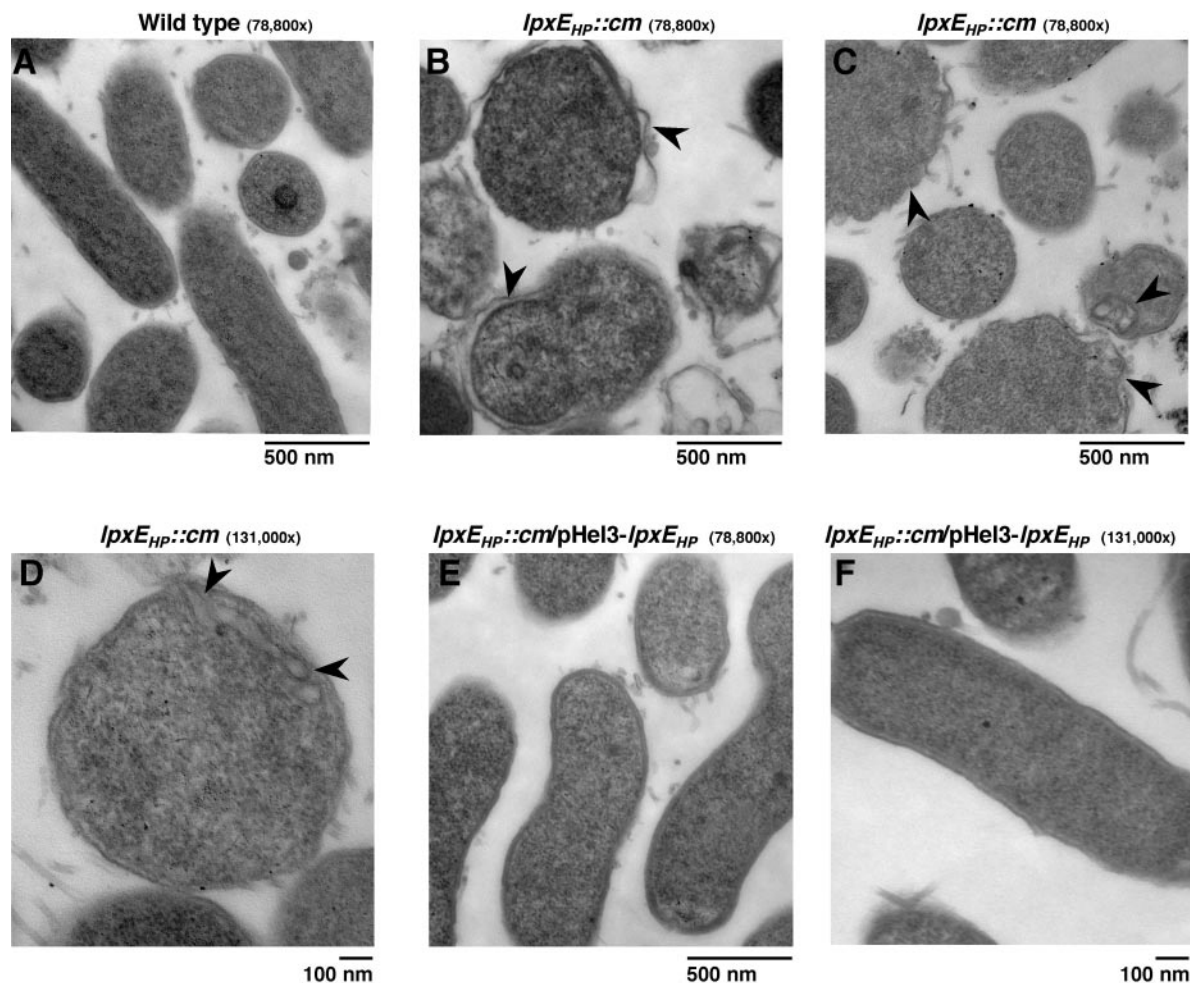


FIG. 7. Transmission electron micrographs of the *H. pylori* 1-phosphatase mutant following exposure to polymyxin. *H. pylori* strains Hp7-91, Hp7-91/*lpxE_{HP}::cm*, and Hp7-91/*lpxE_{HP}::cm* containing pHel3-*lpxE_{HP}* were exposed to 100 μ g/ml of polymyxin for 1 h. The cells were processed as described in Materials and Methods. Changes to the cell envelope structure seen in the *lpxE_{HP}* mutant following exposure to polymyxin are indicated by arrowheads.

that removal of the 4'-phosphate group of *H. pylori* lipid A is also critical for CAMP resistance.

It has long been known that polymyxin-resistant variants of both *S. enterica* serovar Typhimurium and *E. coli* produce lipid A species modified with both pEtN and L-Ara4N. Masking of a lipid A phosphate group with L-Ara4N has been conclusively shown to be critical for CAMP resistance (15, 17, 57). However, the importance of pEtN addition for resistance to CAMPs is not as clearly defined. For example, *Salmonella pmrC* (*eptA*) mutants show only slight increases in susceptibility to killing by polymyxin (less than fivefold) in comparison to the wild type (30, 54). On the other hand, loss of LptA function, the neisserial homolog of EptA, results in more than a 200-fold reduction in polymyxin resistance (61). Other gram-negative pathogens, such as *Vibrio cholerae* (6), *Campylobacter jejuni* (38), and *P. gingivalis* (28), modify the lipid A domain of their LPSs with either one or two pEtN groups. Further investigation of pEtN modification in other pathogens will be required to determine its role in CAMP resistance and pathogenesis.

During infection of the gastric mucosa, *H. pylori* encounters antimicrobial peptides produced by its human host. For example, expression of the human cathelicidin LL-37 (22) and human β -defensin 2 is upregulated in the gastric mucosa of *H. pylori*-infected patients (20, 66). Furthermore, *H. pylori* itself produces a cecropin-like peptide, Hp (2–20), to which it is resistant, that is derived from the amino-terminal part of its ribosomal protein L1 (44). Cecropin has been shown to bind to bis-phosphorylated lipid A (10), and *phoP* mutants of *Salmonella* unable to mask their lipid A phosphate groups show increased sensitivity to cecropin A (47). Therefore, reduction or masking of the phosphate groups of *H. pylori* lipid A should provide resistance to cecropin-like peptides. Indeed, the *H. pylori* 26695 *lpxE_{HP}* mutant producing an unmodified lipid A structure displays complete sensitivity to cecropin A in comparison to the wild type (unpublished results). Since *H. pylori* undergoes “altruistic lysis” in vivo (42), it has been suggested that *H. pylori* cecropin may provide an advantage to the bacterium by inhibiting the growth of competing organisms (44). On the whole, the current work provides initial evidence sup-

porting the importance of lipid A modifications for the survival of *H. pylori* upon exposure to antimicrobial peptides.

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