Mol. Cells, Vol. 10, No. 6, pp. 633–641

Molecules and Cells

Cloning and Characterization of a 22 kDa Outer-Membrane Protein (Omp22) from *Helicobacter pylori*

Jang-Seong Kim, Ji-Hoon Chang, Won-Young Seo, Gum-Ju Yu, Soo-Il Chung, and Jung-Sun Yum*
Mogam Biotechnology Research Institute, Yongin 449-910, Korea.

(Received on June 1, 2000)

***Helicobacter pylori* is a causative agent of gastritis and peptic ulceration in humans. As the first step towards development of a vaccine against *H. pylori* infection, we have attempted to identify protective antigens. A potential target of vaccine development would be a *H. pylori* specific protein, which is surface-exposed and highly antigenic. We identified a 22 kDa outer-membrane protein (Omp22) from *H. pylori*, which was highly immunoreactive. By screening a *H. pylori* genomic DNA library with rabbit anti-*H. pylori* outer-membrane protein antibodies, the *omp22* gene was cloned and 1.4 kb of the nucleotide sequence was determined. One open reading frame, encoding a 179-residue polypeptide, was identified and the amino acid sequence deduced showed homology with peptidoglycan-associated lipoproteins. The sequence was conserved among other *H. pylori* strains. Omp22 protein is expressed as a precursor polypeptide of 179 residues and undergoes lipid modification and cleavage of an 18 amino acid signal peptide to yield a mature protein. Omp22 protein in *H. pylori* as well as recombinant Omp22 protein expressed in *E. coli* was localized into the outer membrane and exposed on the cell surface. Omp22 may have the potential as a target antigen for the development of a *H. pylori* vaccine.**

Keywords: *Helicobacter pylori*; Peptidoglycan-associated Lipoprotein.

Introduction

Helicobacter pylori is a gram-negative, microaerophilic bacterium that colonizes the gastric antrum of the human stomach. Since Marshall and Warren (1984) first isolated and cultured this bacterium from antral biopsy specimens of human stomach, studies on this organism have been actively carried out worldwide. Epidemiological studies have consistently demonstrated that *H. pylori* is a causative agent of active chronic gastritis and peptic ulcers, and it is a primary risk factor for the development of intestinal-type gastric adenocarcinoma (Forman *et al.*, 1994; IARC, 1994; Sipponen *et al.*, 1989). Moreover, this organism is associated with mucosa-associated lymphoid tissue (MALT) and consequently with B-cell MALT lymphomas (Parsonnet *et al.*, 1994); however, the actual mechanism of pathogenesis has not been completely determined. It has been recommended that all *H. pylori* positive patients with peptic ulcers should receive antibiotic treatment for eradication of the bacterium. Although the antibiotic treatment regimens currently used are effective, problems such as the emergence of antibiotic-resistant strains, poor patient compliance, and the high cost of therapy are major drawbacks that limit the efficacy of the chemotherapeutic intervention on a large scale (Malfertheiner, 1993). Furthermore, infections cured with antimicrobial agents in adults do not induce immune protection against reinfection (Schutze *et al.*, 1995); therefore, development of alternative prophylactic and therapeutic measures against *H. pylori* infection are required. Vaccination to prevent (or control) and to eradicate *H. pylori* infection in humans has received increased attention. The identification of protective antigens is one of the major goals of vaccine development.

To be a vaccine target, an antigenic protein of *H. pylori* should be located at the surface of the bacterium to be accessible to the effector immune response of the host and should be expressed, without structural variation, by all strains. In gram-negative bacteria, many surface-exposed proteins are constituents of the outer membrane. The outer membrane is an interface between the environment and the interior of

* To whom correspondence should be addressed.
Tel: 82-31-260-9835; Fax: 82-31-260-9808
E-mail: jsyum@greencross.com

Abbreviations: HRP, horse-radish peroxidase; KCTC, Korean Collection for Type Cultures; MALT, mucosa-associated lympoid tissue; OMP, outer-membrane protein; PAL, peptidoglycan-associated lipoprotein.

the cell (Hancock, 1991). It plays a major role in determining what enters a cell, which molecules are exported from the cell, and how the cell interacts with its environment as well as a structural role. Studies on the outer-membrane structure and proteins therein are important to understand the pathogenesis and immunology of *H. pylori* infection. Although little is known about the outer-membrane architecture of *H. pylori*, several outer-membrane proteins (OMPs) have been identified and characterized to date, such as a family of porin proteins (HopA, HopB, HopC, and HopD), a 31 kDa porin protein (HopE), and a 19 kDa lipoprotein (Doig *et al.*, 1995; Drout *et al.*, 1991; Exner *et al.*, 1995).

This study was carried out to identify surface-exposed, antigenic proteins of *H. pylori*, which may be potential targets for therapy and vaccine development. We screened a genomic expression library of *H. pylori* KCTC0217BP with an antibody raised against *H. pylori* outer-membrane proteins. We report the identification, molecular cloning, and characterization of a novel 22 kDa outer-membrane lipoprotein (Omp22) of *H. pylori*, which shows several characteristics which make it a suitable vaccine antigen candidate.

Materials and Methods

Bacterial strains and growth conditions *H. pylori* strains ATCC43504, ATCC43579, and ATCC43629 were purchased from American Type Culture Collection (ATCC) and eight clinical strains including *H. pylori* KCTC (Korean Collection for Type Cultures) 0217BP were isolated from Korean patients suffering from gastritis, gastric ulcers, and duodenal ulcers. *H. pylori* strains were grown on selective blood agar plates containing 22 g of Columbia blood agar base (Difco)/l, 20 g of tryptic soy agar (Difco)/l, 7% (vol/vol) sheep blood, 10 μg of vancomycin (Sigma)/ml, 300 units of colistin (Sigma)/ml, and 2.5 μg of amphotericin B (Sigma)/ml. Plates were incubated at 37°C for 48–72 h under 10% CO_2.

Fractionation of *H. pylori* proteins To prepare *H. pylori* whole cell lysates, bacterial cells were harvested, resuspended in 10 mM Tris/HCl, pH 7.4, and disrupted with an ultrasonifier (Model B-30, Branson). Intact cells and cell debris were removed by centrifugation at $10{,}000 \times g$ for 20 min. The protein concentration in the supernatant was measured using a BCA protein assay kit (Pierce). The supernatant was ultra centrifuged for 1 h at $100{,}000 \times g$ at 4°C (L8-70 ultracentrifuge, Beckman). The resultant pellet was resuspended in sterile distilled water and was termed the crude membrane protein fraction. One percent sodium lauryl sarkosinate in 7 mM EDTA was added to the crude membrane fraction and incubated for 30 min at 37°C. This suspension was centrifuged at $100{,}000 \times g$ for 1 h at 4°C and the pellet resuspended in 10 mM Tris/HCl, pH 7.4. This Sarkosyl-insoluble fraction was considered to contain OMPs. The crude membrane proteins and the OMPs were analyzed by SDS-PAGE (12% acrylamide) and Western blotting.

Antibody preparation Polyclonal antibody against *H. pylori* was produced in Sprague-Dawley rats. The animals were injected intraperitonealy with a washed suspension of *H. pylori* ATCC43504 cells (10^9 cells per rat) and boosted after 10 d. Blood was collected 2 weeks after the last injection. Polyclonal antibody against the OMPs of *H. pylori* was produced in a rabbit. The animal was immunized with 140 μg of Sarkosyl-insoluble OMPs prepared from *H. pylori* KCTC0217BP and boosted twice after 1 and 4 weeks, and blood was collected 10 d after the last injection. This rabbit anti-OMP preparation was employed for genomic library screening and Western blot analysis. Polyclonal antibody against *H. pylori* Omp22 was produced in a similar manner using 200 μg of purified recombinant Omp22 protein.

Screening of *H. pylori* genomic library The *H. pylori* expression library was constructed in the Lambda Zap II vector (Stratagene) using genomic DNA of *H. pylori* KCTC0217BP. A Gigapack II packaging extract kit (Stratagene) was used for *in vitro* packaging following standard procedures (Sambrook *et al.*, 1989). The titer of the recombinant library was calculated to be 1.25×10^6 PFU/ml by infecting *E. coli* strain XL1-Blue MRF′ cells with aliquots of packaged phage. The amplified library with a titer of 2.5×10^{10} PFU/ml was screened with a 1:2,000 dilution of rabbit anti *H. pylori* OMP antibodies. The antigen–antibody complexes were visualized with HRP-conjugated goat anti-rabbit IgG antibody by using 3′, 3′-diaminobenzidine (DAB) and 0.03% H_2O_2 as the enzyme substrates. Once a positive phage clone was identified, an agar plug containing the plaque was picked, and the phage was eluted. Plaque purity was confirmed by repeating the screening process at least three times.

Cloning and expression of Omp22 protein in ΔpMA vector The entire coding region of the *omp22* gene was amplified by PCR with primers, 5′-GGACTACCATATGAAAAGATCTTCTG-TA-3′ and 5′-GGAATTCTTACTTCACTA ATTTGACATC-3′ according to standard protocols. The amplified DNA fragment of 540 bp was digested with restriction enzymes *Nde*I and *Eco*RI and then ligated into *Nde*I/*Eco*RI-digested ΔpMA, an expression vector which is designed to express a foreign gene under control of the *E. coli araB* promoter (Park *et al.*, 1998; Schleif, 1996). The resulting plasmid was transformed into *E. coli* MC1061 and the recombinant Omp22 protein was expressed under the following conditions. One liter of Luria-Bertani broth containing ampicillin (50 μg/ml) was inoculated with 10 ml of an overnight culture of *E. coli* MC1061 transformant and incubated with shaking at 37°C. When the OD_{600} of the culture reached 0.5, arabinose was added at a final concentration of 1% (w/v). The cells were incubated for an additional 4 h and were then harvested by centrifugation at $8{,}000 \times g$ for 30 min at 4°C. The recombinant protein expression was analyzed by SDS-PAGE and Western blotting.

Site-directed mutagenesis Site-directed mutagenesis to determine the possible cleavage site of Omp22 leader peptide was performed as described by Kunkel (1985). Briefly, pBluescript SK(-) (Stratagene) containing the *omp22* gene was transformed into *E. coli* RZ1032 (*ung*$^-$, *dut*$^-$) and uracil-incorporated single-stranded template DNA was prepared by using

a Wizard M13-kit (Promega). The template DNA was then annealed with a custom-synthesized mutagenic primer 5′-TAGCTGGTGGTAAACAC-3′ after phosphorylation, incubated with T4 DNA polymerase at 37°C for 3 h for the synthesis of the second strand, and ligated with T4 DNA ligase. After treatment with uracil-DNA glycosylase to destroy the uracil-containing original strand *in vitro*, the DNA was transformed into *E. coli* DH5α (*ung*$^+$, *dut*$^+$). The resulting mutation was verified by DNA sequencing.

Labeling of proteins with [^{3}H]palmitate *E. coli* MC1061 containing the wild-type *omp22* gene (pOmp22-Cys) and the mutated *omp22* gene (pOmp22-Gly) were radiolabeled with [^{3}H]palmitic acid. *E. coli* cells carrying each construct were grown in LB broth containing ampicillin (50 μg/ml) and protein expression was induced by 1% arabinose. To exponentially growing cells, [^{3}H]palmitic acid (5 mCi/ml) (DuPont-NEN) was added to a final concentration of 25 μCi/ml, and incubation was continued for 3 h. The cells were collected by centrifugation and washed three times with methanol to remove unincorporated radioactive precursors and noncovalently bound lipids. The dried pellets were resuspended in sample buffer and analyzed by SDS-PAGE and the labeled proteins were visualized by autoradiography.

Flow cytometric analysis *H. pylori* KCTC0217BP cells grown on blood agar plates were suspended in PBS. After washing three times with PBS, 5×10^7 cells were incubated with rabbit anti-Omp22 antibody at 4°C for 50 min. After washing twice with PBS, FITC-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) was added as a secondary antibody and incubated for 50 min at 4°C. The cells were washed twice with PBS to remove unbound antibody. Fluorescence intensities on stained cells were measured using a flow cytometer (FACSCalibur, Beckton Dickenson). The background was determined by staining the cells with FITC-conjugated goat anti-rabbit IgG alone and preimmune serum together with FITC-conjugated goat anti-rabbit IgG. To confirm the binding specificity of the anti-Omp22 antisera, cells stained with rabbit anti-Omp22 which was preincubated with the purified recombinant Omp22 protein were analyzed by flow cytometry under the same condition as described previously.

Nucleotide sequence accession number The nucleotide sequences of *omp22* have been deposited in GenBank under the accession number U75869.

Results and Discussion

Identification of a 22 kDa outer-membrane protein derived from *H. pylori* To identify *H. pylori* specific antigenic proteins, we first analyzed antigenic protein profiles of *H. pylori* strains ATCC43504 and Korean isolate KCTC0217BP by SDS-PAGE and Western blotting using antibodies raised against intact *H. pylori* cells. At least six major protein bands with molecular mass of 65, 60, 57, 46, 29, and 22 kDa were identified by SDS-PAGE. The 22 kDa protein gave a strong signal compared to the others on Western blotting with rat

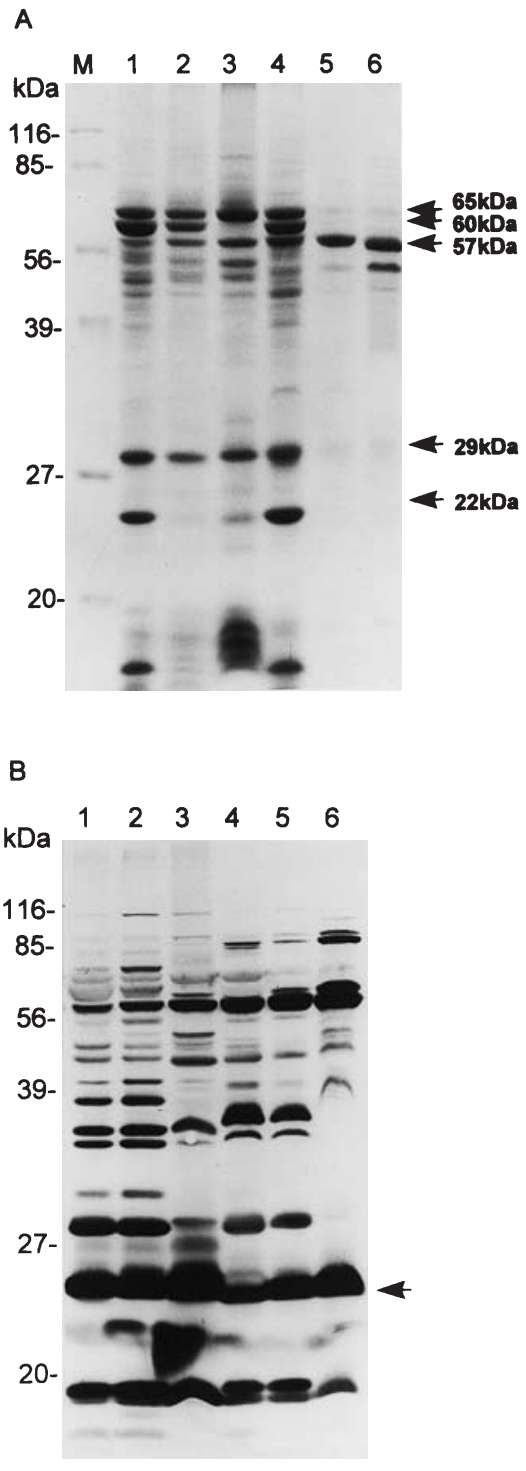

Fig. 1. Identification and localization of a 22 kDa protein of *H. pylori*. Bacterial whole-cell lysates and membrane proteins were analyzed by (**A**) SDS-PAGE (12% acrylamide) and (**B**) Western blotting with a rat anti-*H. pylori* antibody. Lanes contained: whole-cell lysates (lanes 1–2), crude membrane proteins (lanes 3–4), and outer-membrane proteins (lanes 5–6) of *H. pylori* ATCC43504 (lanes 1, 3, 5) and KCTC0217BP (lanes 2, 4, 6). Molecular mass markers (in kilodaltons) are shown on the left. The major protein bands (panel A) and the position of the 22 kDa protein (panel B) are shown on the right.

anti-*H. pylori* antibody (Fig. 1). Moreover, when whole-cell lysates of *H. pylori* KCTC0217BP were analyzed by Western blotting, the same 22 kDa protein was detected in the sera of more than 20 *H. pylori* infected patients but not in noninfected controls (data not shown). To localize this protein, the whole-cell lysates of *H. pylori* KCTC0217BP were fractionated into crude membrane proteins and OMPs (Sarkosyl-insoluble), and the presence of the 22 kDa protein was examined by Western blotting with rat anti-*H. pylori* antibody. The 22 kDa protein was detected in outer-membrane fractions as well as in crude membrane fractions, although this band appeared almost invisible on SDS-PAGE; therefore, this 22 kDa protein was probably an outer-membrane protein which was highly immunoreactive and was thus studied further.

Library screening and cloning of *omp22* gene A genomic library was constructed in Lambda ZapII (Stratagene) using *Hae*III-digested genomic DNA of *H. pylori* KCTC0217BP and screened with anti-*H. pylori* OMP antibody. Approximately 1.6×10^4 plaques were screened and a total of 12 positive plaques were selected. Each clone was transformed into pBluescript SK(-) phagemid by *in vivo* excision (Short *et al.*, 1988). When the protein profile of each clone was analyzed by SDS-PAGE and Western blotting, seven clones were found to express a *H. pylori* specific protein with an estimated molecular mass of 22 kDa. Plasmid DNA was isolated from each clone expressing Omp22 protein and digested with *Eco*RI to give a DNA fragment, ranging from 3.5 to 6 kb in size. The plasmid with the smallest (3.5 kb) DNA fragment inserted was selected for further studies.

To characterize the insert of pOmp22 encoding a 22 kDa OMP, two subclones, pHL and pBBR, were constructed, each of which contained 1.4 and 2.1 kb of the pOmp22 insert, respectively. When the protein profiles of each clone containing pOmp22 or its subclones (pHL and pBBR) were analyzed by Western blotting, pOmp22 and pHL, but not pBBR, expressed a protein with an estimated molecular mass of 22 kDa (Fig. 2). Thus, the region encoding the 22 kDa OMP of *H. pylori* (Omp22) is located on the 1.4-kb fragment of pHL. When either pOmp22 or pHL was expressed in *E. coli,* the 22 kDa protein could be detected in the outer-membrane envelop of *E. coli* (Fig. 2C); therefore, the 1.4-kb fragment of pHL encodes Omp22 of *H. pylori* and includes all the information required for the transport to the outer membrane.

Nucleotide and amino acid sequence analysis of *omp22* gene The entire 1,403-bp nucleotide sequence of the pHL insert was determined and found to contain an open reading frame of 540 nucleotides. The corresponding gene has also been identified and sequenced in *H. pylori* strains 26695 (Tomb *et al.*, 1997) and J99

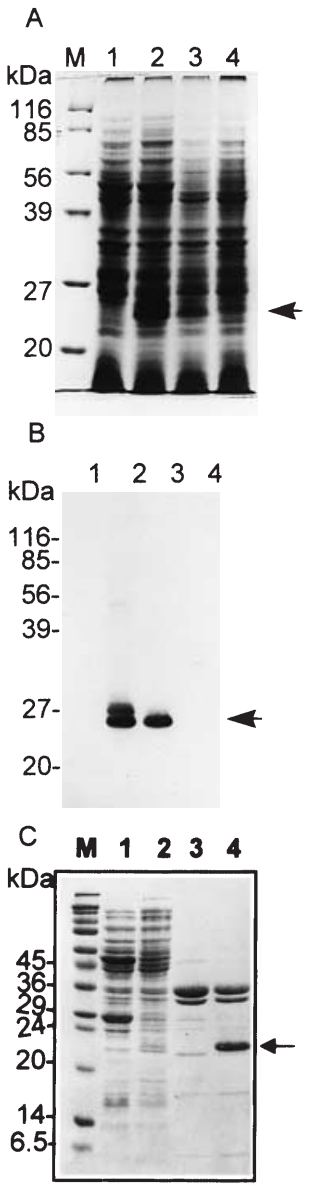

Fig. 2. Omp22 protein produced in *E. coli* cells containing pOmp22 and its subclones, pHL and pBBR. Whole-cell lysates of transformed *E. coli* containing either pOmp22, pHL, or pBBR were analyzed by (**A**) SDS-PAGE (10% acrylamide) and (**B**) Western blotting with a rabbit anti-Omp22. Lane 1, *E. coli* XL1-Blue MRF′; lane 2, *E. coli* XL1-Blue MRF′ containing pOmp22; lane 3, *E. coli* XL1-Blue MRF′ containing pHL; lane 4, *E. coli* XL1-Blue MRF′ containing pBBR. (**C**) Outer-membrane localization of Omp22 protein produced in *E. coli* cells. Whole-cell lysates (lanes 1–2) and outer-membrane proteins (lanes 3–4) of *E. coli* XL1-Blue MRF′ (lanes 1, 3) and *E. coli*, XL1-Blue MRF′ containing pOmp22 (lanes 2, 4) were analyzed by SDS-PAGE (15% acrylamide). Molecular mass markers (in kilodaltons) are shown on the left. The position of the Omp22 protein is indicated by an arrow.

(Alm *et al.*, 1999) and shows 91 and 89% identity, respectively, to the deduced amino acid sequence of our isolate. This open reading frame showed signif-

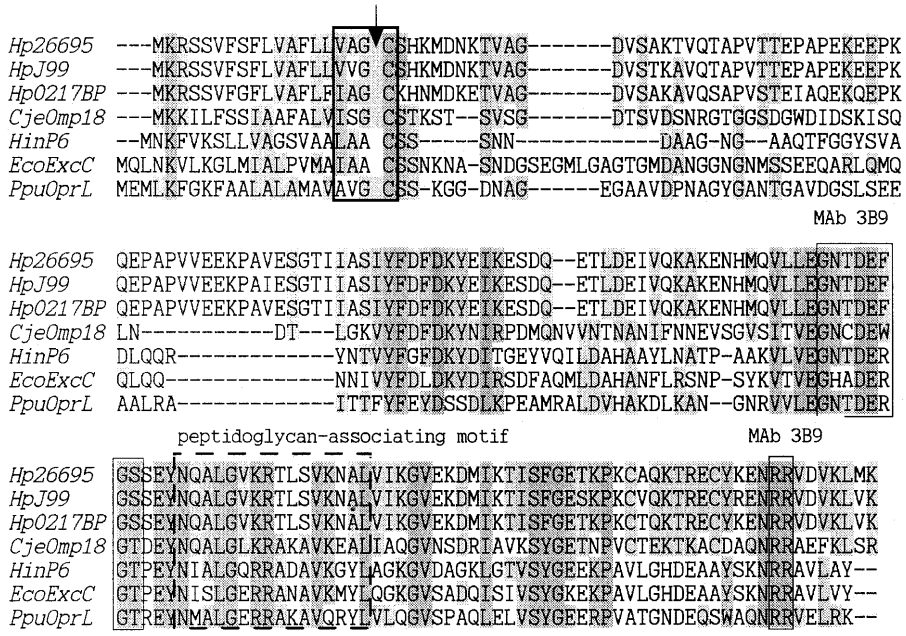

Fig. 3. Multiple amino acid sequence alignment of the *H. pylori* Omp22 with other bacterial PALs. Sequence alignment was generated by the ClustalX (1.8) program. Dark shading shows the identity of the amino acids, while the darkness of the shade represents the degree of similarity. The lipoprotein consensus sequence is shown with a box. The arrow indicates the signal peptide cleavage site. The proposed peptidoglycan-associating α-helical consensus motif and MAb 3B9-binding epitope are shown by boxes. Hp26695, *H. pylori* 26695 (AE000619); HpJ99, *H. pylori* J99 (AE001533); Hp0217BP, *H. pylori* KCTC0217BP presented in this study (U75869); CjeOmp18, *C. jejuni* Omp18 (U47617); HinP6, *H. influenzae* P6 (M19391); EcoExcC, *E. coli* ExcC (X65796); PpuOprL, *P. putida* OprL (CAA52294).

icant sequence homology with the bacterial peptidoglycan-associated lipoproteins (PALs) (Fig. 3), such as *Brucella abortus* Omp16 (Tibor *et al.*, 1994), *Campylobacter jejuni* Omp18 (Konkel *et al.*, 1996), *E. coli* ExcC (Lazzaroni and Portalier, 1992), *Haemophilus influenzae* P6 (Nelson *et al.*, 1988), and *Pseudomonas putida* OprL (Rodriguez-Herva *et al.*, 1996), as demonstrated by an amino acid sequence homology search with the BLAST network service at the National Center for Biotechnology Information (Altschul *et al.*, 1990).

In general, bacterial lipoproteins are synthesized with a signal peptide at their N-termini. Lipid modification and subsequent proteolytic processing to yield a mature lipoprotein take place, and then the localization of the mature lipoprotein to either the inner or the outer membrane follows. The N-terminal amino acid sequence of the *H. pylori* Omp22 showed features typical of a bacterial lipoprotein signal peptide (Fig. 3). The potential signal peptide of 18 amino acid residues is composed of two positively charged residues, Lys at the second and Arg at the third positions, followed by a central hydrophobic domain. The carboxyl terminus of the putative signal peptide contains a sequence (Ile-Ala-Gly-Cys), similar to a consensus tetrapeptide sequence (Leu-X-Y-Cys; X and Y are predominantly neutral small amino acids) which constitutes the recognition site for the modification and processing enzymes for bacterial prolipoproteins (Wu and Tokunaga, 1986). Since the cleavage occurs between the third residue and glyceride-cysteine of the consensus, this sequence defines the junction between the signal peptide and the mature protein. Although Leu and Cys at the first and the fourth position, respectively, of the consensus sequence are believed to be invariable, it appears that Leu at this position can be replaced by either Ala, Ile, or Val (Fig. 3).

Interestingly, the amino acid residue next to the lipid-modified Cys (+2 position) at the N-terminus of a mature lipoprotein is thought to function as a sorting signal (Yamaguchi *et al.*, 1988). Aspartic acid at this position has been proposed to make lipoproteins specific to the inner membrane, whereas other residues, typically serine, have been assumed to direct lipoproteins to the outer membrane. It has been reported that outer-membrane-directed lipoproteins in *E. coli* generally utilize the LolA-LolB system, in a sorting signal-dependent manner, for efficient release from the inner membrane and subsequent localization to the outer membrane (Yokota *et al.*, 1999). Whether the LolA-LolB-like system is also utilized in *H. pylori* is not known. Only the LolA homologue, and not the LolB homologue, was found in the complete genome sequence of *H. pylori*. In the *H. pylori* Omp22 from strain KCTC0217BP, Lys was located at the +2 position, whereas Ser was located at the +2 position in the proteins from strains 26695 and J99. Although Lys at the +2 position is not commonly found, given that

Omp22 expressed in *H. pylori* KCTC0217BP is well localized to the outer membrane, it would appear that both Ser or Lys at the +2 position can function as a sorting signal for outer-membrane localization.

A high degree of amino acid identity between *H. pylori* Omp22 and other bacterial PALs was also observed at the C-terminal end, which is required for the binding of PAL to the peptidoglycan layer. The peptidoglycan-associating α-helical consensus motif, $NX_2LSX_2RAX_2VX_3L$ (X, a variable amino acid), identified in the C-terminus has been proposed to be directly involved in the noncovalent association of a protein with peptidoglycan (Koebnik, 1995; Lazzaroni and Portalier, 1992). The sequence motif ^{122}NQALGVKRTLSVKNAL^{137} is found at the C-terminus of Omp22 of *H. pylori*, where the Gly residue is substituted for the Ser residue at the fifth position and the Thr residue is substituted for the Ala residue at the ninth position of the consensus motif. Gly replaced by Ser is also found in other bacterial PALs (Fig. 3).

All the sequence characteristics described here suggest that *H. pylori* Omp22 is a PAL and this was confirmed experimentally by the following approaches.

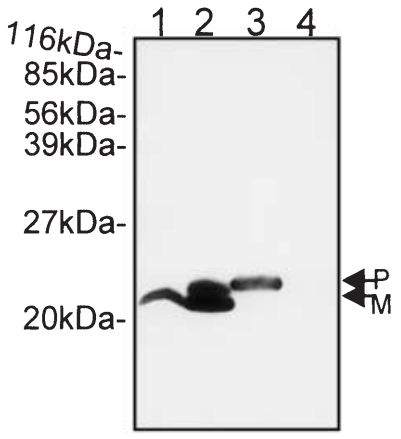

Fig. 4. Western blot analysis of the wild-type and the mutant Omp22 proteins. Whole-cell lysates were separated by SDS-PAGE (15% acrylamide) and reacted with a 1:2,000 dilution of rabbit anti-Omp22 antibody. Lane 1, *H. pylori* KCTC 0217BP; lane 2, *E. coli* MC1061/pOmp22-Cys; lane 3, *E. coli* MC1061/pOmp22-Gly; lane 4, *E. coli* MC1061. Molecular mass markers (in kilodaltons) are shown on the left. The position of the precursor (P) and mature (M) Omp22 proteins are indicated by arrows.

Cleavage of the signal peptide to produce a mature Omp22 On the basis of the amino acid sequence analysis, the *H. pylori* Omp22 protein was predicted to be expressed as a prolipoprotein of 179 amino acids, and the leader sequence of 18 amino acids was processed by a signal peptidase II-like enzyme, resulting in a mature Omp22 protein of 161 amino acids with a predicted molecular mass of 18,050 Da. To experimentally confirm this, we performed site-directed mutagenesis at the putative cleavage site (^{19}Cys) for the signal peptidase II following the method of Kunkel (1985). The substitution of the guanine for the thymidine at nucleotide position +55 results in an amino acid change to Gly from Cys. The entire coding region of both the wild-type and the mutant *omp22* genes was amplified by PCR and cloned into the expression vector ΔpMA, resulting in two constructs containing the wild-type (pOmp22-Cys) and the mutant (pOmp22-Gly) *omp22* genes. Each of these constructs was transformed into *E. coli* MC1061. Following arabinose induction, whole cell proteins of each of the transformants were separated by SDS-PAGE (15% acrylamide) and the Omp22 protein expressed in each construct was analyzed by Western blotting with anti-*H. pylori* Omp22 antibody (Fig. 4). From the *E. coli* MC1061 containing pOmp22-Cys, a 22 kDa protein was expressed which was the same size as the mature Omp22 protein expressed in *H. pylori*. A trace of the precursor Omp22 protein was also observed. In contrast, a 24 kDa protein was expressed in the *E. coli* MC1061 containing pOmp22-Gly. The size difference of 2 kDa corresponds to the size of the putative signal peptide of 18 amino acids, suggesting that the signal peptide is not cleaved in the mutant Omp22 and, thus, the Cys residue in the lipoprotein consensus constitutes the signal peptide cleavage site.

Posttranslational lipid modification The molecular mass of the Omp22 protein expressed in *H. pylori* and the recombinant Omp22 protein expressed in *E. coli* both appeared to be approximately 22 kDa in size on SDS-PAGE. This is larger than the predicted molecular mass of approximately 18 kDa for the mature Omp22 protein. This size discrepancy and the presence of a consensus modification and processing site for prolipoprotein suggest that posttranslational lipid modification may occur.

In most lipoproteins, two fatty acid residues are linked by ester linkages and one fatty acid residue is linked by an amide linkage to the N-terminal glycerylcysteine. The most prevalent fatty acid species attached to bacterial lipoproteins is palmitic acid and thus artificial labeling with palmitate is a useful way to determine if a protein is a lipoprotein. When *E. coli* MC1061 containing either wild-type (pOmp22-Cys) or mutant *omp22* gene (pOmp22-Gly) was grown with [^{3}H]palmitic acid, a [^{3}H]palmitic acid-labeled protein, 22 kDa in size, was observed only in the *E. coli* cells containing wild-type *omp22* gene (Fig. 5). The 24 kDa protein was not detected on the autoradiogram. Lipid modification was completely abolished by amino acid replacement, Cys to Gly, at amino acid position 19, indicating that lipid attachment occurred at the Cys residue.

The effects of lipidation on the physicochemical, structural, antigenic, and immunogenic properties of

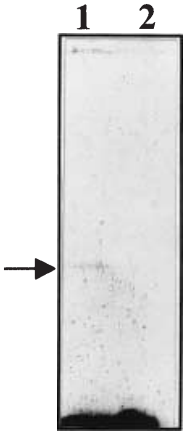

Fig. 5. [^{3}H]palmitic acid radiolabeling of recombinant Omp22 expressed in *E. coli*. Autoradiogram of SDS-PAGE (15% acrylamide) of whole-cell lysates of *E. coli* transformants cultured in the presence of [^{3}H]palmitic acid. Lane 1, *E. coli* MC1061/pOmp22-Cys; lane 2, *E. coli* MC1061/pOmp22-Gly. The position of [^{3}H]palmitic acid-labeled Omp22 is indicated by an arrow.

a protein are not fully understood and experimental results reported to date are controversial. Erdile *et al.* (1993) reported that mice immunized with the OspA lipoprotein were protected against challenge with infectious *Borrelia burgdorferi*, whereas lipid-free OspA did not induce a protective response, indicating that post-translational lipid attachment is a critical determinant of the immunogenicity of lipoproteins. In contrast, Green *et al.* (1990) reported that the lipid component of *H. influenzae* P6 protein was not required for either immunogenicity or elicitation of functional antibodies. Although both the lipid-free recombinant P6 protein and its native counterpart elicited comparable protective immune responses, some physicochemical and immunological properties were different (Yang *et al.*, 1997). The isoelectric point of recombinant P6 was more acidic and exhibited less secondary structure than that of the native protein, suggesting that the lack of acylation of recombinant P6 could be in part responsible for its misfolding or instability. Recombinant P6 was less immunogenic than native P6 possibly owing to the presence of conformational B-cell epitopes expressed on native P6 but not recombinant P6. Similarly, when lipidated Omp22, expressed either in *H. pylori* or in *E. coli*, and nonlipidated recombinant Omp22 were analyzed simultaneously by Western blotting, lipidated Omp22 always reacted with higher intensity than nonlipidated Omp22. This result suggests that the lipid moiety of Omp22 may play an important role in its immunogenicity.

Conservation of Omp22 in *H. pylori* strains The prevalence of the *omp22* gene and its expression in different *H. pylori* strains were investigated. We performed PCR with primers 5′-GCGGTTCAAAGTGCGCCTGTT-3′ and 5′-TTACTTCACTAATTTGACATC-3′ with genomic DNA from ten different *H. pylori* strains as templates. A 540-bp internal fragment of the *omp22* gene was amplified from all of the strains tested (Fig. 6A). The nucleotide sequence of the amplified *omp22* gene from each strain was determined and compared to other strains. The nucleotide sequence homology of the amplified *omp22* gene among strains ranged from 96 to 100%, which suggested that the *omp22* gene is highly conserved among *H. pylori* strains, although a deletion of 12 nucleotides was found in *H. pylori* MBRI#18 (data not shown).

Expression of the Omp22 protein in *H. pylori* strains described previously was also examined by Western blot analysis with rabbit anti-Omp22. Protein bands of 22 kDa were strongly detected in all the strains tested, with the exception of *H. pylori* MBRI#18, where a smaller band was observed (Fig. 6B). These results indicate that the Omp22 protein and its gene are highly conserved in *H. pylori* strains.

Omp22 is a surface-exposed protein We used flow cytometry to determine if the *H. pylori* outer-membrane protein, Omp22, is exposed to the bacterial cell surface (Fig. 7). Given that only the surface-exposed proteins of the intact cells are able to bind with the corresponding antibody, we stained intact *H. pylori* cells with rabbit anti-Omp22, followed by FITC-conjugated anti-rabbit IgG. The fluorescence intensity was measured to count the antibody-bound cells. Compared to the background determined from cells stained with preimmune serum (Fig. 7, trace B), the fluorescence intensity of cells stained with rabbit anti-Omp22 was significantly increased (Fig. 7, trace C), suggesting that the antibody against Omp22 bound to the bacterial cell surface. This

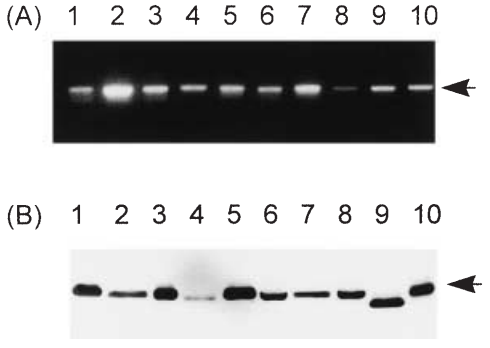

Fig. 6. Conservation of the *omp22* gene and its expression among *H. pylori* strains. (**A**) PCR amplification of *omp22* gene fragment among *H. pylori* strains and (**B**) Western blot analysis of whole-cell lysates of *H. pylori* strains with a rabbit anti-Omp22. *H. pylori* strain in each lane is (1) ATCC43629, (2) ATCC43504, (3) MBRI#25, (4) MBRI#38, (5) MBRI#6, (6) MBRI#2, (7) MBRI#23, (8) MBRI#8, (9) MBRI#18, and (10) KCTC0217BP. PCR-amplified *omp22* gene and Omp22 protein expressed in each strain are indicated by arrows.

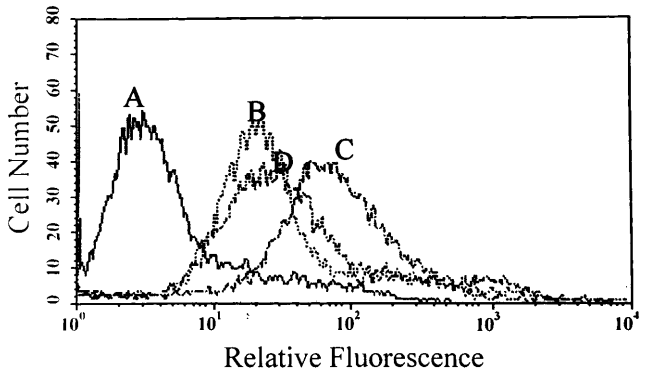

Fig. 7. Flow cytometric analysis of the cell surface exposure. *H. pylori* cells were stained with (**A**) FITC-conjugated goat anti-rabbit IgG alone (negative control); (**B**) preimmune serum (background control); (**C**) rabbit anti-Omp22 antibody; (**D**) rabbit anti-Omp22 preincubated with purified recombinant Omp22 protein (for competitive inhibition). FITC-conjugated goat anti-rabbit IgG was used as a secondary antibody.

anti-Omp22 antibody binding was competitively inhibited by purified Omp22 protein. When Omp22 antibody was preincubated with the purified recombinant Omp22 protein, the fluorescence intensity was decreased to that of cells stained with the preimmune serum (Fig. 7, trace D), which indicates that anti-Omp22 antibody binding to the *H. pylori* cell surface is Omp22-specific. Cell-surface localization of the *H. pylori* Omp22 protein was also confirmed by a radioiodination experiment. When surface proteins of *H. pylori* KCTC0217BP and *E. coli* cells, containing either pOmp22 or pBluescript SK(-), were labeled with NaI^{125} (DuPont-NEN) using IodoBead (Pierce), a protein band with a molecular weight of 22 kDa was radiolabeled in both *H. pylori* KCTC0217BP and *E. coli* cells containing pOmp22, but not in the *E. coli* cells containing pBluescript SK(−) (data not shown). These results strongly indicate that Omp22 is localized on the *H. pylori* cell surface.

PALs have been reported to play diverse functions, including the maintenance of the cell envelope integrity (Lazzaroni and Portalier, 1992; Rodriguez-Herva *et al.*, 1996). Since the function of *H. pylori* Omp22 is not known at this time, attempts to construct an isogenic mutant strain by allelic exchange mutagenesis were performed to elucidate the function of Omp22 but were unsuccessful. Given that an isogenic mutant targeted for the *fli*D gene was successfully constructed under the same experimental conditions (Kim *et al.*, 1999), our result may reflect an essential role for the *omp22* gene in cell viability.

We demonstrated in this study that *H. pylori* Omp22 is a highly immunogenic lipoprotein, which is conserved in *H. pylori* strains and is present on the bacterial surface. Such characteristics are essential requirements for an antigen to be used in a *H. pylori* vaccine. Since bacterial PALs in various gram-negative bacteria have been reported to be highly immunogenic and conserved, they might be useful as immunogens to protect humans and animals from disease caused by gram-negative pathogens. In fact, P6 of *H. influenzae* is suggested to be an important antigen for the induction of protective immunity (Green *et al.*, 1993; Murphy *et al.*, 1992). *H. influenzae* P6 contains a surface-exposed epitope recognized by monoclonal antibody (MAb) 3B9, which binds to a conformational determinant composed of two discontinuous regions, ^{87}GNTDERGT^{94} and ^{147}RR^{148} (Bogdan and Apicella, 1995; Spinola *et al.*, 1996). Although we have not demonstrated that MAb 3B9 binds to *H. pylori* Omp22, the carboxyl terminus of Omp22 contains two similar sequences, ^{111}GNTDEFGS^{118} and ^{171}RR^{172}. Homologous sequences with similar spacing between the two regions are also observed in other bacterial PALs (Fig. 3). Since *H. pylori* Omp22 was shown to be surface-exposed, these sequences may represent a surface-exposed epitope.

In summary, we found a highly immunogenic OMP of *H. pylori*, Omp22, which shows significant sequence similarity with PALs of other bacteria. By mutagenesis studies, we demonstrated that Omp22 is expressed as a prolipoprotein, and lipid modification and processing of the signal peptide occurs at the lipoprotein consensus sequence (Ile-Ala-Gly-Cys) to produce the mature lipoprotein. We also demonstrated that Omp22 of *H. pylori* is highly conserved and present on the cell surface, which may make it a good candidate for detection by a diagnostic kit or to be used in the development of a vaccine.

Acknowledgments This work was supported by the Korea Green Cross Corporation.

References

Alm, R. A., Ling, L-S. L., Moir, D. T., King, B. L., Brown, E. D., Doig, P. C., Smith, D. R., Noonan, B., Guild, B. C., deJonge, B. L., Carmel, G., Tummino, P. J., Caruso, A., Uria-Nickelsen, M., Mills, D. M., Ives, C., Gibson, R., Merberg, D., Mills, S. D., Jiang, Q., Taylor, D. E., Vovis, G. F., and Trust, T. J. (1999) Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**, 176–180.

Altschul, S. F., Gish, W., Miller, W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Bogdan, J. A. and Apicella, M. A. (1995) Mapping of a surface-exposed, conformational epitope of the P6 protein of *Haemophilus influenzae*. *Infect. Immun.* **63**, 4395–4401.

Doig, P., Exner, M. M., Hancock, R. E. W., and Trust, T. J. (1995) Isolation and characterization of a conserved porin protein from *Helicobacter pylori*. *J. Bacteriol.* **177**, 5447–5452.

Drout, E. B., Denoyel, G. D., Boude, M., Wallano, E., Andujar, M., and de Montclos, H. P. (1991) Characterization of an immunoreactive species specific 19-kilodalton outer membrane protein from *Helicobacter pylori* by using a monoclonal antibody. *J. Clin. Microbiol.* **29**, 1620–1624.

Erdile, L. F., Brandt, M. -A., Warakomski, D. J., Warakomski, D. J., Westrack, G. J., Sadziene, A., Barbour, A. G., and Mays, J. P. (1993) Role of attached lipid in immunogenicity of *Borrelia burgdorferi* OspA. *Infect. Immun.* **63**, 81–90.

Exner, M. M., Doig, P., Trust, T. J., and Hancock, R. E. W. (1995) Isolation and characterization of a family of porin proteins from *Helicobacter pylori*. *Infect. Immun.* **63**, 1567–1572.

Forman, D., Webb, P., and Parsonnet, J. (1994) *Helicobacter pylori* and gastric cancer. *Lancet* **343**, 243–244.

Green, B. A., Metcalf, B. J., Quinn-Dey, T., Kirkley, D. H., Quataert, S. A., and Deich, R. A. (1990) A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both non-typeable and type b *H. influenzae*. *Infect. Immun.* **58**, 3272–3278.

Green, B. A., Vazquez, M. E., Zlotnick, G. W., Reape, G. Q., Swarts, J. D., Green, I., Cowell, J. L., Bluestone, C. D., and Doyle, W. J. (1993) Evaluation of mixtures of purified *Haemophilus influenzae* outer membrane proteins in protection against challenge with nontypeable *H. influenzae* in the chinchilla otitis media model. *Infect. Immun.* **61**, 1950–1957.

Hancock, R. E. W. (1991) Bacterial outer membranes: evolving concepts. *ASM News* **57**, 175–182.

IARC. (1994) Schistosomes, liver flukes and *Helicobacter pylori*. *IARC Monogr. Eval. Carcinog. Risks Hum.* **61**, 177–220.

Kim, J. S., Chang, J. H., Chung, S. I., and Yum, J. S. (1999) Molecular cloning and characterization of the *Helicobacter pylori fliD* gene, an essential factor in flagellar structure and motility. *J. Bacteriol.* **181**, 6969–6974.

Koebnik, R. (1995) Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some bacterial cell surface proteins. *Mol. Microbiol.* **16**, 1269–1270.

Konkel, M. E., Mead, D. J., and Cieplak Jr, W. (1996) Cloning, sequencing and expression of a gene from *Campylobacter jejuni* encoding a protein (Omp18) with similarity to peptidoglycan-associated lipoproteins. *Infect. Immun.* **64**, 1850–1853.

Kunkel, T. A. (1985) Rapid efficient site-directed mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**, 488–492.

Lazzaroni, J. C. and Portalier, R. (1992) The *exeC* gene of *Escherichia coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (PAL). *Mol. Microbiol.* **6**, 735–742.

Malfertheiner, P. (1993) Compliance, adverse events and antibiotic resistance in *Helicobacter pylori* treatment. *Scand. J. Gastroenterol.* **196 (Suppl.)**, 34–37.

Marshall, B. J. and Warren, J. R. (1984) Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **I**, 1311–1314.

Murphy, T. F., Nelson, M. B., and Apicella, M. A. (1992) The P6 outer membrane protein of nontypeable *Haemophilus influenzae* as a vaccine antigen. *J. Infect. Dis.* **165 (Suppl. 1)**, S203–S205.

Nelson, M. B., Apicella, M. A., Murphy, T. F., Vankeulen, H., Spotila, L. D., and Rekosh, D. (1988) Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. *Infect. Immun.* **56**, 128–134.

Park, D., Kang, I., Kim, H., Chung, K., Kim, D., and Yun, Y. (1998) Cloning and characterization of novel disintegrins from *Agkistrodon halys* venome. *Mol. Cells* **8**, 578–584.

Parsonnet, J., Hansen, S., Rodriguez, L., Gelb, A. B., Warnke, R. A., Jellum, E., Orentreich, N., Vogelman, J. H., and Frieman, G. D. (1994) *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* **330**, 1267–1270.

Rodriguez-Herva, J. J., Ramos-Gonzalez, M., and Ramos, J. L. (1996) The *Pseudomonas putida* peptidoglycan-associated outer membrane lipoprotein is involved in maintenance of the integrity of the cell envelope. *J. Bacteriol.* **178**, 1699–1706.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning; a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schleif, R. (1996) The L-arabinose operon; in *Escherichia coli and Salmonella: cellular and molecular biology*, Neidhardt, F. C., Curtiss III, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E. (eds.), 2nd ed., pp. 1473–1481, ASM press, Washington, DC.

Schutze, K., Hentschel, E., Dragosics, B., and Hirschl, A. M. (1995) *Helicobacter pylori* reinfection with identical organisms: transmission by the patients' spouses. *Gut* **36**, 831–833.

Short, J. M., Fernandez, J. M., Sorge, J. A., and Huse, W. D. (1988) Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res.* **15**, 7583–7600.

Sipponen, P., Seppala, K., Aarynen, M., Helske, T., and Kettunen, P. (1989) Chronic gastritis and duodenal ulcer: a case-control study on risk of coexisting duodenal or gastric ulcer in patients with gastritis. *Gut* **30**, 922–929.

Spinola, S. M., Hiltke, T. J., Fortney, K., and Shanks, K. L. (1996) The conserved 18,000-molecular-weight outer membrane protein of *Haemophilus ducreyi* has homology to PAL. *Infect. Immun.* **64**, 1950–1955.

Tibor, A., Weynants, V., Denoel, P., Lichtfouse, B., DeBolle, X., Saman, E., Limet, J. N., and Letesson, J. J. (1994) Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kilodalton outer membrane protein of *Brucella abortus* with similarity to pal lipoproteins. *Infect. Immun.* **62**, 3633–3639.

Tomb, J. F., White, O., Kerlavage, A. R., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Goldek, A., McKenney, K., Fitzgerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Utterback, T. R., Peterson, J. D., Kelley, J. M., Cotton, M. D., Weidman, J. M., Fujii, C., Bowman, C., Watthey, L., Wallin, E., Hayes, W. S., Borodovsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C. (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**, 539–547.

Wu, H. C. and Tokunaga, M. (1986) Biogenesis of lipoproteins in bacteria. *Curr. Top. Microbiol. Immunol.* **125**, 127–157.

Yamaguchi, K., Yu, F., and Inouye, M. (1988) A single amino acid determinant of the membrane localization of lipoproteins in *E. coli*. *Cell* **53**, 423–432.

Yang, Y. -P., Munson Jr, R. S., Grass, S., Chong, P., Harkness, R. E., Gisonni, L., James, O., Kwok, Y., and Klein, M. H. (1997) Effect of lipid modification on the physicochemical, structural, antigenic and immunoprotective properties of *Haemophilus influenzae* outer membrane protein P6. *Vaccine* **15**, 976–987.

Yokota, N., Kuroda, T., Matsuyama, S., and Tokuda, H. (1999) Characterization of the LolA–LolB system as the general lipoprotein localization mechanism of *Escherichia coli*. *J. Biol. Chem.* **274**, 30,995–30,999.