Protein Hpn: Cloning and Characterization of a Histidine-Rich Metal-Binding Polypeptide in *Helicobacter pylori* and *Helicobacter mustelae*

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Received 20 January 1995/Returned for modification 7 March 1995/Accepted 6 April 1995

Helicobacter pylori is a human gastrointestinal pathogen involved in gastritis, duodenal ulcers, and gastric neoplasia. This microorganism produces large amounts of a urease which, like all known ureases, has nickel in the active site. We have identified a protein in clinical isolates of *H. pylori* and an identical protein in the ferret pathogen *Helicobacter mustelae* that strongly binds Ni^{2+} and Zn^{2+} . This protein has been named Hpn to emphasize its origins in *H. pylori* and its affinity for nickel. The encoding *hpn* gene, cloned and expressed in *Escherichia coli* ER1793, has an open reading frame (180 bp) that specifies a protein with a calculated molecular mass of 7,077 Da and with the same amino-terminal sequence as that of wild-type Hpn. The deduced sequence of Hpn consists of 60 amino acids, of which 28 (47%) are histidines. The *hpn* gene does not map with the urease gene cluster on the *H. pylori* chromosome. An Hpn-negative, isogenic *H. pylori* strain, generated by *hpn* gene deletion and grown on blood agar, had the same urease activity that wild-type cells did. Thus, the role of Hpn in helicobacters is unknown.

Helicobacter pylori is a gram-negative bacterium that has been implicated as an essential cofactor or direct cause of gastritis, duodenal ulcers, and possible gastric neoplasia (4, 32, 35, 36, 38). Often eradication of this agent by antimicrobial drugs is necessary to interrupt a clinical cycle of recurrent gastritis or ulcer, providing evidence for a role in pathogenesis. The precise means by which *H. pylori* injures the gastric mucosa has not been established, although several virulence factors in this organism have been identified (6, 12, 17, 26). Primary and secondary effects of the inflammatory reaction may promote mucosal injury by recruiting inflammatory cells, inducing local production of cytokines, reducing blood flow, or decreasing mucin and other protective proteins.

H. pylori produces large amounts of the enzyme urease (9, 13, 20, 21, 34), which is essential for colonization of the gastric epithelium in experimental models of infection (11). Generation of ammonia by urea cleavage may neutralize the low gastric pH around colonizing microorganisms, although studies with a piglet model of *Helicobacter* infection showed that urease was essential regardless of whether the animal host secreted gastric acid. Recombinant urease-negative strains were incapable of colonizing the gastric mucosae of either normal or achlorhydric piglets (10). Thus, the role of urease in colonization, infection, and gastric injury remains uncertain.

An invariant finding among plant and bacterial ureases is the presence of a nickel ion at or near the enzyme-active site (19, 33, 41, 47), and the presence of nickel in the urease of *H. pylori* has been directly demonstrated (20). Examining the mechanisms by which *H. pylori* acquires nickel in the gastric environment, we have consistently detected a low-molecular-weight, metal-binding protein in clinical isolates of this microorganism. Here we report the identification, isolation, and cloning of this

protein, which we have named Hpn to emphasize its origins in *H. pylori* and its avidity for nickel. Hpn consists of 60 amino acids, of which 28 are histidines. Rapid isolation of Hpn by metal affinity chromatography was made possible by exploiting its binding to nickel, since the protein selectively bound to columns packed with nickel ions in the form of nickel(II)-nitrilotriacetic acid-agarose (NTA-agarose). We also report the presence of a protein with an identical sequence in *Helicobacter mustelae* (14), a natural pathogen of ferrets. We present evidence that Hpn is not essential for cell growth in vitro and that the urease activity in an organism in which the encoding *hpn* gene has been deleted is normal.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. (i) *H. pylori*. Six clinical isolates from human infections, designated All, Gly, PCC 6, Sin, Win, and Leu, were provided by David Cave; *H. pylori* 1011-4 and *H. mustelae* were provided by James Fox.

Helicobacter strains were cultured on *Campylobacter* agar Skirrow (Difco) plates supplemented with 10% defibrinated sheep's blood (Remel, Lenexa, Kans.) in chambers that had been made microaerobic by the CampyPak system (BBL). Liquid cultures in the same microaerobic environment were grown in *Brucella* broth (Difco) supplemented with 0.2% cyclodextrin and vancomycin (5 µg/ml) in borosilicate tubes or in 100- to 500-ml borosilicate bottles on a platform oscillated at 200 rpm. Medium was prepared with deionized water, without using metal-free reagents, culture vessels, or other equipment for cell or protein processing.

(ii) *Escherichia coli*. Strain ER1793 [F^- *fhuA2* $\Delta(\text{lacZ})\text{r1}$ *supE44* *e14*⁻ (McrA^-) *trp-31* *his-1* *rpsL104* (Str^r) *xyl-7* *mtl-2* *metB1* $\Delta(\text{mcrC-mrr})114::\text{IS10}$] (23), used as the host strain for recombinant plasmids, was grown in L broth or on L agar plates. For selection of *E. coli* strains harboring plasmids, the following antibiotic concentrations were used in media: ampicillin, 200 µg/ml; kanamycin, 30 µg/ml. The plasmid cloning vector used for expression of the *Helicobacter* library was pBluescript SK(+) (Stratagene). Plasmids pHPN1 and pHPN2 were clones containing the *hpn* gene from the *H. pylori*/pBluescript SK library. Plasmid pHPN3, a derivative of pHPN1, contained the *hpn* gene but lacked the upstream *H. pylori* DNA sequence. Plasmid pHPN2KO was pHPN2 with the *hpn* gene deleted and contained a kanamycin resistance gene (Fig. 1).

Cell fractionation. Cells harvested at 24 h from Skirrow blood agar plates were washed with phosphate-buffered saline (PBS) containing 1.3 mM KH_2PO_4 , 11 mM Na_2HPO_4 , and 140 mM NaCl, placed in 50 mM Na_2HPO_4 (pH 8) containing 10 mM β -mercaptoethanol, and then ruptured by using a French press at 23,000

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FIG. 1. Restriction maps of plasmids pHPN1 and pHPN2, used to clone the *hpn* gene of *H. pylori*, and pHPN2KO, used for deletion of the *hpn* gene (discussed in Materials and Methods). The *hpn* gene is shown as a black box. The vector is pBluescript SK (pSK). S/B, *Sau3A1/BamHI*; E, *EcoRI*; H, *HindIII*; BII, *BglII*; BX, *BstXI*; N, *NcoI*; Kan, kanamycin resistance gene. The direction of transcription (feathered arrow) and primers for amplification of pHPN2 to construct pHPN2KO (closed arrows) are shown.

lb/in². After centrifugation (1 h at 100,000 × *g* and 4°C), pellet membranes were solubilized by shaking for 1 h in 6 M guanidine-HCl (pH 8.0) at room temperature. Supernatants were also made 6 M with guanidine-HCl, and the pH was adjusted to 8.0 with Tris base. The distribution of Hpn in membrane and supernatant fractions was determined by recovering protein by NTA-agarose chromatography, by inspecting preparations on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and by protein assays (Bio-Rad).

Protein isolation. *H. pylori* Leu was used for identification and purification of Hpn. Cells from five confluent agar plates that had been cultured for 24 h were suspended and washed once in PBS. Pellets were stored frozen at -20°C until used. Thawed pellets were resuspended in 7 ml of buffer A (all lettered buffers discussed subsequently are those specified by the manufacturer of NTA-agarose resin [QIAGEN Inc., Chatsworth, Calif.]). Buffer A consisted of 6 M guanidine-HCl, 100 mM Na₂HPO₄, and 10 mM Tris-HCl (pH 8.0). Suspended cells were shaken at room temperature for 1 h and centrifuged at 10,000 × *g* for 20 min at 4°C, and supernatants containing extracted *Helicobacter* proteins were subjected to NTA-agarose chromatography (QIAGEN Inc.).

Columns containing 0.5 ml of packed Ni-NTA resin were equilibrated with buffer B consisting of 8 M urea, 10 mM Tris-HCl, and 100 mM sodium phosphate, adjusted to pH 8.2. Supernatants were passed through this resin, and columns were washed with 10 volumes of buffer B, 10 volumes of buffer C (buffer B [pH 6.3]), and then 10 volumes of buffer D (buffer B [pH 5.9]). Hpn was then recovered by elution with 5 ml of buffer E (buffer B [pH 4.5]), supplemented with 0.1 M EDTA and 0.1 M dithiothreitol. Eluted proteins were analyzed for Hpn on SDS-15% polyacrylamide gels stained with Coomassie blue. As needed, these proteins were concentrated with Centricon membranes (Amicon Corporation, Lexington, Mass.).

Protein characterization. Structural studies of Hpn were done with protein isolated by elution from polyacrylamide gel fragments. Concentrated nickel resin eluates (about 1 mg of protein per ml) were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE). Gel segments containing Hpn were excised (using molecular mass markers as guides), suspended in 25 mM Tris-192 mM glycine buffer (pH 8.8) (transfer buffer lacking methanol), and placed into dialysis tubing with a 3,500-kDa cutoff. Then gels were subjected to electroelution for 2 h at 4°C and 100 V on a Bio-Rad mini-transblot apparatus, and the eluted protein was dialyzed overnight at 4°C against large volumes of 0.05% SDS-5.0 mM ammonium bicarbonate-HCO₃. Dialyzed samples were transferred to microcentrifuge tubes and concentrated 20-fold in a SpeedVac apparatus, after which protein was precipitated by adding 9 volumes of acetone. After storage overnight at -20°C, each sample was centrifuged at 4°C and the pellet containing purified wild-type Hpn was washed with cold acetone and dried in air.

To prepare Hpn protein for trypsin digestion, purified Hpn was resuspended in 50 μl of 8 M urea-0.40 M ammonium bicarbonate (pH 8.0) and reduced with 45 mM dithiothreitol. After being heated at 50°C for 15 min, the material was cooled to 25°C and alkylated in 0.1 M iodoacetamide. Water (140 μl) was added to 60 μl of protein solution. At pH 9.3, the protein was hydrolyzed by incubation with 0.25 μg of bovine trypsin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 37°C for 24 h. SDS-PAGE analysis and silver staining were used to assess the degree of hydrolysis. Samples incompletely hydrolyzed were adjusted to pH 8.5 with HCl and further digested for 16 h with a second addition of 0.25 μg of trypsin.

Tryptic peptides were separated by high-pressure liquid chromatography (HPLC) (Vydac C₁₈ resin) on an acetonitrile gradient (0 to 60%) in 0.1%

trifluoroacetic acid, and selected peaks were analyzed for their amino-terminal sequences by automatic Edman degradation by using a model 477A pulsed-liquid protein sequencer (Applied Biosystems, Foster City, Calif.) at the Protein Analysis Facility of Tufts University School of Medicine. The amino-terminal amino acid sequences of Hpn proteins blotted onto polyvinyl membranes (28) were also determined at the Protein Analysis Facility.

Preparation and processing of DNA. Plasmid DNA extraction, restriction enzyme digestions, ligations, and transformations in *E. coli* were performed according to the methods of Maniatis et al. (31), unless otherwise noted. Genomic DNA from *H. pylori* was purified according to the method of Spiegelhalter et al. (42).

For construction of an *H. pylori* gene library, chromosomal DNA from strain Leu was partially digested with *Sau3A1* to generate fragments of 2 to 9 kb. After density gradient fractionation, DNA fragments were ligated into plasmid pBlue-script SK that had been digested with *BamHI* and treated with shrimp phosphatase (United States Biochemical, Cleveland, Ohio). Ligated DNA was used to transform competent ER1793 cells by electroporation (13), and transformants were selected by growth on L agar medium containing 200 μg of ampicillin per ml and 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG).

Cloning of Hpn in *E. coli*. Approximately 6,000 transformants, obtained as described above, were screened for Hpn by the Western blot (immunoblot) method of Hall et al. (18). The only modification was electroelution of membranes at 50 V for 30 min to remove SDS.

Antibody preparation and library screening. Antibodies to Hpn were isolated from serum pooled from two patients who had recently been treated for *H. pylori* infections. Purified Hpn from strain Leu was transferred from polyacrylamide gels to Immobilon P membranes in 25 mM Tris-192 mM glycine transfer buffer (with no methanol) at 25 V for 50 min. After unoccupied sites on membranes were blocked with bovine serum albumin (BSA) (10g/liter), membrane segments (approximately 1 cm²) containing Hpn were removed and soaked in a solution containing 24 ml of Tris-BSA and 1 ml of patient serum. After being shaken for 3 h at room temperature, membrane fragments were washed five times with PBS-0.5% Tween 20, and antibodies were eluted by acidification in 20 ml of 0.1 M glycine containing 0.15 M NaCl (pH 2.6). Eluates were restored to pH 7 to 8 by dropwise addition of 1 M Tris (pH 8) and finally diluted to 50 ml with PBS containing 0.5% Tween 20. Before use, antibody solutions were absorbed at 4°C with a sonicate of *E. coli* ER1793.

Clarified *E. coli* colonies on membranes (46) were screened with affinity-purified anti-Hpn antibodies by incubation in antibodies for 3 h at room temperature. Then membranes washed with 0.5% Tween 20 in PBS were incubated with goat anti-human immunoglobulin G conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.), and Hpn-positive colonies were identified by developing membranes with *p*-nitroblue tetrazolium chloride (0.4 mM)-5-bromo-4-chloro-3-indolylphosphate (BCIP; 0.4 mM).

DNA sequencing. Double-stranded plasmid DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (39) with Sequenase 2 (United States Biochemical) and Bluescript oligonucleotide primers (Stratagene). Deduced amino acid sequences were compared with other known sequences by using a BLAST server at the National Center for Biotechnology, Bethesda, Md.

Southern hybridization. Restriction endonuclease-digested DNAs were fractionated by electrophoresis on 1% agarose gels, transferred to positively charged nylon membranes (Boehringer Mannheim Biochemicals), and hybridized at 68°C with fluorescein-tagged DNA probes by random priming with the fluorescein-11-dUTP Gene Images labelling system (Amersham Corp., Arlington Heights, Ill.) according to the manufacturer's protocols.

PCR. *Helicobacter* genomic DNA was amplified by *Taq* polymerase (Boehringer Mannheim Biochemicals) with a single block thermal cycler (Ericomp, San Diego, Calif.). Oligonucleotide primers (Genosys Biotechnologies, Inc., The Woodlands, Tex.) were specific for the *hpn* gene. Reaction cycle profiles were as follows: DNA denaturation at 94°C for 5 min, followed by 30 cycles consisting of 1-min denaturation at 94°C, 2-min annealing at 55°C, and 2-min extension at 72°C.

For amplification of plasmid DNA, the method of Barnes was followed (2) and the following DNA polymerase mix was used: 23 U of KlenTaq 1, 3' exonuclease-negative polymerase (Ab Peptides, St. Louis, Mo.) and 0.16 U of DNA polymerase (Stratagene). Amplification consisted of 24 cycles, with a cycling profile of 30-s denaturation at 95°C and 12-min annealing-extension at 75°C.

Metal-binding assays. Wild-type Hpn from agar plate cultures of strain Leu and recombinant Hpn from *E. coli* ER1793 cultured overnight in 10 ml of L broth were purified on NTA-agarose columns as described above. Proteins were mixed with an equal volume of Laemmli sample buffer containing 0.1 M dithiothreitol, heated for 7 min at 100°C, fractionated by SDS-PAGE on 15% polyacrylamide gels, and electroblotted onto nitrocellulose membranes at 25 V for 50 min with Tris-glycine transfer buffer (containing no methanol). Membranes were probed with ⁶⁵NiCl₂ (3.7 μM; specific activity, 12 mCi/mg of Ni), ⁶⁵ZnCl₂ (5 μM; specific activity, 1.4 mCi/mg of Zn), or ¹⁰⁹CdCl₂ (8 nM; specific activity, 150 mCi/mg of Cd) as described by Makowski et al. (30), with radionuclides from NEN, DuPont Co., Boston, Mass. Binding of ¹⁰⁹Cd²⁺, ⁶⁵Zn²⁺, and ⁶⁵Ni²⁺ to Hpn was visualized by autoradiography (29). Then membranes were stained for proteins with amido black. Metal blot competition assays (30) were performed to determine if Zn²⁺, Cu²⁺, Co²⁺, Cd²⁺, or Mn²⁺ (100 μM [as chlorides]) added to

FIG. 2. Coomassie blue stain of *H. pylori* proteins resolved on an SDS-12% polyacrylamide gel. Lanes 2 and 3, lysate of *H. pylori* 1011-4 and its purified Hpn, respectively; lanes 5 and 4, strain Leu lysate and its Hpn, respectively; lane 1, molecular mass markers (in kilodaltons).

^{63}Ni probing medium at 27-fold molar excess [compared with Ni^{2+}]) reduced the binding of $^{63}\text{Ni}^{2+}$ to recombinant Hpn on Western blots.

Construction of plasmid pHPN3. Plasmid pHPN3 was constructed as follows: a 309-bp *Eco*RI fragment of pHPN1 was ligated with pBluescript SK that had been digested with *Eco*RI. The orientation of the *hpn* gene in pBluescript SK was the same as that in pHPN1.

Deletion of the *hpn* gene. An *hpn* gene deletion on plasmid pHPN2 was constructed by PCR (2). A 6.7-kb PCR product was amplified from pHPN2 (Fig. 1) by using primer 1 (5'-CGCAGGCCCTTTCATGGTGTGCCATGATGACTCC TTTGATTAA-3'), which is complementary to nucleotides 465 to 433, and primer 2 (5'-CGAAGGCCTACGAGTAATATCGGTGTGGCTAGGGCAA CTTG-3'), which corresponds to nucleotides 626 to 658 of the nucleotide sequence of the *hpn* gene (the orientations of primers are shown in Fig. 3). Each primer had a *Stu*I restriction site added at its 5' end to facilitate cloning. The amplification product, which lacked the *hpn* gene sequence from nucleotides 466 to 625, was digested with endonuclease *Stu*I and ligated with a 1.3-kb *Hinc*II fragment containing the Tn903 kanamycin resistance gene, derived from plasmid pRI133 (received from R. Isberg). The resulting plasmid, designated pHPN2KO, contained only the first 15 and last 5 bp of this gene and was introduced into *E. coli* ER1793 by transformation, and lysates of kanamycin-resistant colonies were screened by NTA binding and SDS-PAGE for expression of Hpn. QIAGEN plasmid DNA from this Hpn-negative transformant was prepared, and 30 μg was used for electroporation of *H. pylori* Leu (13). Colonies cultured on Skirrow blood plates for 24 h were harvested with SOC broth (13) and then plated on blood agar containing 30 μg of kanamycin per ml. After 48 h of incubation at 37°C, individual kanamycin-resistant colonies were purified and analyzed for Hpn by Western blot analysis of cell lysates. Chromosomal DNA was prepared from an Hpn-negative isolate, designated strain *H. pylori*/Hpn(-), and examined by Southern hybridization.

Assay of urease activity. For urease assay, cells were harvested from agar plates and washed in PBS. Pellets were resuspended in 30 ml of ice-cold 20 mM Na_2HPO_4 (pH 6.8) containing 1 mM EDTA and 5 mM β -mercaptoethanol and ruptured by two passes through a precooled French pressure cell (23,000 lb/in 2). Lysates were centrifuged at 100,000 $\times g$ (1 h at 4°C), and supernatants were assayed on the same day for urease activities. Urease activity was assayed by the Berthelot reaction at room temperature with urea substrate (110 mM) (40).

Jackbean urease (Sigma) was used as the positive control, and ammonium chloride was used as the standard. Protein concentrations were estimated (Bio-Rad), with BSA as the standard.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank under accession number U26361.

RESULTS

Identification of wild-type Hpn. Sonicates of *H. pylori* Leu applied to nickel(II)-NTA-agarose affinity columns yielded a protein, designated Hpn, with an apparent molecular mass of 16 kDa on reduced SDS-12% polyacrylamide gels (Fig. 2). Western blots of affinity-purified, wild-type Hpn developed with convalescent-patient serum showed a protein of the same size as that on stained SDS-polyacrylamide gels (data not shown). All sera from five other patients infected with *H. pylori* reacted with the 16-kDa protein, but the titers were variable (range, 1:400 to 1:1,000 [vol/vol]). As these sera were used solely as reagents for identification of Hpn, the possible absence of anti-Hpn antibodies in sera from uninfected patients has not been formally examined.

N-terminal sequence analysis of wild-type Hpn. Hpn was subjected to Edman-type amino-terminal analysis, which provided limited sequence information. Trypsin hydrolysates of this protein separated by HPLC yielded several fragments, of which two were also examined by partial amino-terminal sequence analysis, giving the sequence information shown in Table 1. Comparison of these results with the deduced recombinant Hpn amino acid sequence is discussed below.

Cloning of the *H. pylori hpn* gene in *E. coli* and sequence analysis of the recombinant protein. A genomic library of *H. pylori* Leu in the Stratagene pBluescript SK vector was expressed in *E. coli* ER1793 and screened by modified Western blot analysis for the presence of the 16-kDa protein. Three positive colonies, among 6,000 examined, on subculture produced a 16-kDa protein that was indistinguishable on polyacrylamide gels and by Western blot analysis from wild-type Hpn. One clone was selected to isolate plasmid pHPN1; DNA from this plasmid was characterized by restriction enzyme analysis (Fig. 1) and shown to contain a 710-bp insert of helicobacter DNA. The DNA sequence of the complete insert in pHPN1 was determined by double-stranded nucleotide sequencing and found to contain a 180-bp open reading frame (ORF) with the deduced amino acid sequence shown in Fig. 3. Three separate regions of this deduced sequence were identical to *H. pylori* Hpn protein sequences, as determined by Edman degradation (Table 1). By Edman analysis, purified, recombinant Hpn from *E. coli* (Table 1) had an amino-terminal sequence identical to that deduced from the gene (Fig. 3), which was identical to the amino-terminal sequence of Hpn from *H. pylori*. This strongly suggests that the cloned gene encodes Hpn. The predicted amino-terminal methionine was absent from purified proteins in both cases. Further support for the 180-bp ORF encoding Hpn is suggested by the fact that pHPN3, which contains a 309-bp subclone of pHPN1, also encoded full-length Hpn (data not shown).

The DNA sequence upstream of the *hpn* gene contains two

TABLE 1. Amino-terminal amino acid sequences of *H. pylori* wild-type Hpn, recombinant Hpn, and two trypsin fragmentation peaks of wild-type Hpn

Type of Hpn	Sequence ^a
Wild type.....	-A/M-H/K-H-E-E-
Recombinant.....	-A-H-H-E-E-Q-H-G-G-H-
Trypsin fragmentation peak 1.....	-?-H-H-E-E-Q-H-G-G-H-H-H/N-H-?-H-T/E-
Trypsin fragmentation peak 2.....	-?-?-H-H-Q-E-E-G-?-?-H-G-H-H-E-

^a The single-letter amino acid code is used. x/y, ambiguity between two possible residues at that position; ?, residue at that position could not be defined. The complete deduced amino acid sequence of Hpn is given in Fig. 3.

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1   D L N D S L D C A K K L L K L L N G I K S K V N L I L F N P H E G S
   gatctaaacgacagcttggtgctgctaaaaaacttttaaaacttttaaacggcattaatccaaagtgaacttgattttattcaaccgcgcgaaggct

101  K F E R P S L E S A R M F A D F L N S K G L L C T I R E S K A L D
   ctaaatttgagcgccccagtttagagagtgccagaatgtttgaggatttttaaaactctaaaggcttattatgcaccattagagagtctaaagccttgga

201  I E A A C G Q L R E K K L S Q Q I *
   tattgaagcggcttgcgggcaattgaggagaaaaaactctcccaaaaatttgaaaacttttttgggtgtttgtcttttttctaattgggggtgttg
                                     -35                               -10
301  ttttaatacagcgataattcaaaatttaggggatgattgattaaaaattagcactaaattttctttcatttaacaattaattagattttatattgtagaatg
                                     M A H H E E Q H G G H H H H H H H
401  gaattctagccagtgagctagaatttaatttttaatacaaaaggagtcacatcatggcacaccatgaagaacagcagcggtcatcaccaccatcaccacca
                                     T H H H H Y H G G E H H H H H H S S H H E E G C C S T S D S H H Q
501  cacacaccaccaccactatcacggcggtgaacaccaccatcaccaccacagctctcatcatgaagaaggttgttgagcactagcgatagtcacatcatcaa
   E E G C C H G H H E *
601  gaagagggttgctgccacgggcatcacgagtaatatcggtgtggctaggggcaacttgactgtctctagcggttttaattttattttctctcaaatatat
                                     → ←
701  tcaatcaattggatc

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FIG. 3. Nucleotide and deduced amino acid sequences of the *hpn* gene of *H. pylori* Leu. The putative ribosome binding site and -10 and -35 promoter elements are underlined. The deduced amino acid sequence of Hpn is shown in boldface single-letter code. The deduced amino acid sequence of a partially sequenced upstream gene (described in the text) is shown in lightface. *, stop codon. Arrows define the putative transcription terminator region.

partial ORFs. The first ORF, which is in the same reading frame as the *hpn* gene, extends from nucleotide 1 to a stop codon beginning at nucleotide 253. This ORF has 37% identity with a region of the *E. coli* chromosome which lies between the essential *ndk* gene and *gcpE* and encodes a hypothetical 43.1-kDa protein of unknown function (1). The second ORF is oriented in the opposite direction of the *hpn* gene and extends over 175 bp. No significant homology between this ORF and any protein in data banks was found.

Primer extension experiments (unpublished data) with a primer complementary to nucleotides 534 to 517 of the *hpn* gene sequence showed that the *hpn* mRNA transcript begins at nucleotide 406 or 407. The putative -10 and -35 promoter regions in Fig. 3 are based on this information.

Downstream of the *hpn* gene is a potential RNA stem-loop structure which extends from nucleotides 644 to 671 and has a calculated ΔG of -13.9 kcal/mol (1 cal = 4.184 J). This region of mRNA is followed by a stretch of uridines, suggesting that it is a transcriptional terminator.

The putative initiation codon in the *hpn* gene is preceded by an apparent Shine-Dalgarno sequence (Fig. 3). Assuming that this is the translational start site, the amino acids in Hpn have a calculated molecular mass of 7,077 Da. The mobility of this protein from SDS-12 to 15% PAGE analysis did not agree with this value. However, on 20% acrylamide gels, this protein migrated to a location consistent with the calculated molecular mass. Aberrant migrations at lower acrylamide concentrations are unexplained.

Hpn has several notable features, most of which are consistent with high metal-binding capacity. Among its 60 amino acids are 28 histidines (i.e., 47%), including two stretches of 6 and 7 contiguous histidine residues. The sequence also has two paired cysteine residues. Hpn is likely to be highly hydrophilic, as predicted by a Kyte-Doolittle hydrophobicity plot (25) (data not shown); Hpn has no positively charged amino acids nor any tryptophan, leucine, isoleucine, phenylalanine, or proline residues, all of which are hydrophobic. There are indications of internal repeats. For example, the stretch E-E-G-C-C occurs twice, and the longer segment H-H-E-E-G-C-C requires only a single third-position nucleotide change to correspond to H-Q-E-E-G-C-C.

Localization of Hpn in *H. pylori*. Hpn was absent from the cell-free culture supernatants of *H. pylori* cells. In cells fractionated as described above, Hpn was equally distributed between membrane and soluble fractions.

Metal-binding characteristics of wild-type and recombinant Hpn. When purified samples of wild-type Hpn and recombinant Hpn were fractionated by SDS-PAGE, blotted onto nitrocellulose, and probed with $^{63}\text{Ni}^{2+}$, $^{65}\text{Zn}^{2+}$, and $^{109}\text{Cd}^{2+}$, autoradiograms showed intense binding of $^{63}\text{Ni}^{2+}$ and $^{65}\text{Zn}^{2+}$ to both forms of Hpn but only faint binding of $^{109}\text{Cd}^{2+}$ (Fig. 4 and 5). These experiments were repeated three times with consistent results. Five divalent metal ions were tested for competition assays. Western blots from SDS-PAGE fractionations of recombinant Hpn were exposed to $^{63}\text{Ni}^{2+}$ (3.7 μM) under standard conditions, except that radioprobe media contained 27-fold-higher concentrations of ZnCl_2 , CuCl_2 , CoCl_2 , CdCl_2 , or MnCl_2 , compared with that of $^{63}\text{Ni}^{2+}$. Under these conditions, Zn^{2+} completely blocked the binding of $^{63}\text{Ni}^{2+}$ to Hpn; the abilities of metal ions to compete with $^{63}\text{Ni}^{2+}$ for Hpn binding on Western blots were ranked as follows: $\text{Zn}^{2+} > \text{Co}^{2+} > \text{Cu}^{2+} > \text{Cd}^{2+} > \text{Mn}^{2+}$. At a 27-fold molar excess, Mn^{2+} did not significantly reduce the binding of $^{63}\text{Ni}^{2+}$ to recombinant Hpn. These experiments were also repeated three times with consistent results.

Identification of Hpn in other *Helicobacter* strains. Proteins corresponding to Hpn were subsequently identified in sonicates of all five additional *H. pylori* strains and the single *H. mustelae* strain examined. In each case, this protein was obtained from these cells by NTA-agarose chromatography. The DNA region homologous to *hpn* in these six strains was amplified by using primers 5'-CGAGGATCCGTTGGTTTAA TCAAGCG-3' and 5'-CGATCTAGAAAGTTGCCCTAGC CACA-3', which included restriction sites at their 5' ends, and each PCR product was sequenced (double stranded) after being cloned into plasmid pBluescript SK. All *H. pylori* strains had nucleotide sequences that specified amino acid sequences identical to that of *hpn*, although two of these isolates had conservative (third-base-pair) variations in several codons. *H. mustelae* chromosomal DNA had a nucleotide sequence identical to that of *H. pylori* Leu *hpn*.

FIG. 4. (A) Amido black stain of a nitrocellulose membrane on which the following proteins were blotted from SDS-15% polyacrylamide gels: purified Hpn from *H. pylori* (lane 2) and *E. coli* ER1793/pHPN1 (lane 4). Identical treatments of lysates from *H. pylori*/Hpn(-) (lane 3) and *E. coli* ER1793/pHPN2KO (lane 5) contain no Hpn. Lane 6, *E. coli* lysate containing pBluescript SK with no insert; lane 1, molecular mass markers (in kilodaltons). (B) Autoradiogram of membrane in panel A after exposure to $^{63}\text{NiCl}_2$ (described in Materials and Methods), showing metal binding to both wild-type Hpn and recombinant Hpn and no binding to Hpn-negative mutants of these cells or to pBluescript SK. $^{63}\text{Ni}^{2+}$ also binds to marker proteins BSA (67 kDa) and carbonic anhydrase (30 kDa).

Construction of an Hpn deletion mutant of *H. pylori* and analysis of its urease activity. An Hpn deletion mutant of *H. pylori* Leu, designated *H. pylori*/Hpn(-), lacking the *hpn* ORF, was constructed by transformation with plasmid pHPN2KO (Fig. 1). Southern blot analysis showed that a new restriction site had been introduced into this mutant strain, confirming deletion of the *hpn* gene (Fig. 6). Strain *H. pylori*/Hpn(-) produced no Hpn, as analyzed by SDS-polyacrylamide gel staining, and no protein corresponding to Hpn was found on protein blots of this gel probed with $^{63}\text{Ni}^{2+}$ (Fig. 4). The urease activity of this strain was indistinguishable from that of wild-type *H. pylori*, 8.0 and 8.7 $\mu\text{mol NH}_3 \text{ min}^{-1} \text{ mg}^{-1}$ of protein, respectively.

DISCUSSION

This paper reports the identification and primary structure of Hpn, a histidine-rich protein found in cell lysates of both *H. pylori* and *H. mustelae*. This protein was isolated and partially purified in a single step by applying bacterial cell lysates to nickel affinity columns. The deduced amino acid sequence of Hpn has several interesting features that explain its ability to bind metal ions with high avidity. These include multiple charged amino acids, two cysteine pairs, and large amounts of histidine, including stretches of six and seven contiguous His

FIG. 5. (A) Amido black stain of a nitrocellulose membrane on which the following proteins were blotted from SDS-15% polyacrylamide gels: purified Hpn from *H. pylori* (lanes 2) and *E. coli* ER1793/pHPN1 (lanes 3). Lanes 1, molecular mass markers (in kilodaltons). (B and C) Autoradiograms of membrane in panel A after exposure to $^{65}\text{ZnCl}_2$ (B) and $^{109}\text{CdCl}_2$ (C), showing strong binding of $^{65}\text{Zn}^{2+}$ and weak binding of $^{109}\text{Cd}^{2+}$ to Hpn (the photograph does not capture the very weak cadmium bands seen). The marker labelled by cadmium in panel C is bovine lactalbumin.

residues. The presence of a histidine residue at the third position from the deduced N terminus mimics the Asp-Ala-His tripeptide which is known to be a strong nickel-binding site at the N terminus of human serum albumin (5, 45). However, the N-terminal sequence of the isolated recombinant protein we obtained lacks the specified terminal Met residue, for reasons as yet undetermined. The precise metal-binding site and molar capacity for various metals, including nickel, have not yet been established. Hpn is soluble and likely to be highly hydrophilic, since 37 of its 60 amino acids are charged. The only hydrophobic residues specified by the *hpn* gene are the amino-terminal methionine and the adjacent alanine.

The function of Hpn in bacterial cells is unknown. Because of its avidity for nickel, it was thought that a phenotypic characteristic of *H. pylori* *hpn* deletion mutants might be a reduced

FIG. 6. Autoradiogram of chromosomal DNA from wild-type *H. pylori* Leu (lane 1) and the Leu *hpn* deletion mutant (lane 2). DNA was restricted with *Ava*I and probed with fluorescein-11-dUTP-labelled pHPN1, showing that an additional kanamycin-related restriction site is present in this mutant strain. Molecular mass markers are lambda DNA digested with *Hind*III.

capacity to express active urease. However, this was not the case, since Hpn deletion mutants grew normally in laboratory media, with urease activities that were approximately the same as that of isogenic wild-type cells. Alternatively, since urease activity has been reported to be 80 and 30% reduced in the presence of 1 mM Zn^{2+} and 10 mM Ni^{2+} , respectively, and slightly reduced in the presence of EDTA (37), a metal-binding protein within or surrounding *Helicobacter* cells may act to increase, or at least preserve, urease activity. The Hpn deletion phenotype may become manifest only when mutant cells are cultured under more limited conditions, such as those encountered in vivo, particularly if the concentration of metal ions has an influence on colonization or urease activity and distribution. Although we are presently examining possible interaction between Hpn and products of the urease cluster that direct assembly of the active enzyme, as well as the enzyme itself, the encoding *hpn* gene is not homologous to any of the genes in the urease gene cluster and mapping of the gene (performed by Douglas Berg) shows that it is distant from the urease gene cluster on the *H. pylori* chromosome. Although Hpn does not have typical structural characteristics of zinc finger proteins, a role in gene regulation or other interaction with bacterial DNA cannot be excluded.

In vitro binding of Ni^{2+} and Zn^{2+} was observed when wild-type or recombinant Hpn was tested by a Western blot assay that selectively visualizes proteins with high affinities for specific radionuclides (3, 16, 30). The exceptional avidities of Hpn for Ni^{2+} and Zn^{2+} binding were confirmed by competition with other divalent transition metals in metal blot competition assays. The stoichiometry and dissociation constants for Ni^{2+} and Zn^{2+} binding to Hpn have not been determined, but we plan to measure these parameters by equilibrium dialysis. To establish the native metal content of Hpn, it will be necessary to isolate Hpn from wild-type *H. pylori* under conditions that circumvent metal contamination.

The deduced amino acid sequence of Hpn is homologous to those of several histidine-rich proteins of other bacteria (27, 43, 44, 48) and protozoans, but these homologies are largely based on an abundance of histidines and/or cysteine pairs (as in protein WHP of *E. coli* [48]) and provide no clear insights into genetic origins or functional relationships. Several bacterial proteins, including bacterial CO dehydrogenase, methyl-coenzyme M reductase, and hydrogenases, are known to require nickel for biological activity; hydrogenase activity has been reported for *H. pylori* (8), but no metal dependence or structural information has been described. The *hypB* gene in the nitrogen-fixing microorganism *Bradyrhizobium japonicum* and a similar gene in *E. coli* encode histidine-rich proteins that have roles in hydrogenase synthesis (15). Histidine-rich proteins may have a role in the pathogenesis of malaria (24). Proteins rich in histidines can have high buffering capacities that allow microorganisms to adapt to pH extremes. Not only are the pK_a values of histidine-imidazole groups close to physiological pH, but the buffering capacities of histidine-rich proteins in the reduced intracellular environment are enhanced by an abundance of ionizable amino acids (e.g., glutamic and aspartic acids) and cysteine. We have not precisely localized Hpn in *H. pylori* cells cultured in laboratory media, but this protein was associated only with cell constituents, with none found in spent culture medium.

A role for Hpn in colonization or tissue injury can be defined only after the wild type and deletion mutants have been tested in appropriate models of infection, such as gnotobiotic piglets (10, 11). Hpn in the single *H. mustelae* strain examined was identical to that in *H. pylori*, offering an opportunity to study the role of Hpn in the ferret model (14). This also allows

examination of a role for Hpn in gastroduodenal injury, since metal binding may generate short-lived but highly reactive hydroxyl or oxygen intermediates (through Fenton-type reactions) (22) that are capable of damaging host tissue proteins, lipids, or DNAs; a role for reactive intermediates in human gastric injury has already been proposed (7). Clearly, if metal binding by Hpn or any other *Helicobacter* protein plays a role in injury, then that protein must be present at or near the mucosa in vivo and an appropriate metal must be available. In this regard, it is interesting that some of the foods rich in nickel (e.g., tea, chocolate, and nuts) have been implicated in acid-peptic disease.

ACKNOWLEDGMENTS

We thank Anne Kane, Jiazhou Qiu, Kalai Mathee, Adam Antonijczuk, Carol Nichols, Karen Wetterhan, Dean Wilcox, Harry Mobley, and Elizabeth Raleigh for assistance and/or advice with various phases of this research. We also thank Douglas Berg for mapping the *hpn* gene on the *H. pylori* chromosome.

This work was supported by National Institutes of Health grants NIDR 06977, NIAID AI20337, and ES-05331-05 and by the Tufts-NEMCH NIH GRASP Core Center for Digestive Diseases (grant DK34928).

REFERENCES

1. Baker, J., and J. Parker. 1994. Sequence and characterization of the *Escherichia coli* genome between the *ndk* and the *gcpE* genes. FEMS Microbiol. Lett. **121**:293–296.
2. Barnes, W. M. 1994. PCR amplification of up to 35kb DNA with high fidelity and high yield from lambda bacteriophage templates. Proc. Natl. Acad. Sci. USA **91**:2216–2220.
3. Beck, B. L., D. C. Henjum, K. Antonijczuk, O. Zaharia, G. Korza, J. Ozols, S. M. Hopper, A. M. Barber, and F. W. Sunderman, Jr. 1992. pNiXa, Ni^{2+} -binding protein in *Xenopus* oocytes and embryos, shows identity to Ep45, an estrogen-regulated hepatic serpin. Res. Commun. Chem. Pathol. Pharmacol. **77**:3–16.
4. Blaser, M. J. 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. **161**:626–633.
5. Callan, W. M., and F. W. Sunderman, Jr. 1973. Species variations in the binding of $^{63}\text{Ni}(\text{II})$ by serum albumin. Res. Commun. Chem. Pathol. Pharmacol. **5**:459–472.
6. Cover, T. L., and M. J. Blaser. 1992. Purification and characterization of the vacuolating toxin from *Helicobacter pylori*. J. Biol. Chem. **267**:10570–10575.
7. Davies, G. R., N. Banatvala, C. E. Collins, M. T. Sheaff, Y. Abdi, L. Clements, and D. S. Rampton. 1994. Relationship between infective load of *Helicobacter pylori* and reactive oxygen metabolite production in antral mucosa. Scand. J. Gastroenterol. **29**:419–424.
8. Davison, A. A., D. J. Kelly, and C. L. Clayton. 1994. Molecular cloning and sequencing of a *Helicobacter pylori* hydrogenase. Am. J. Gastroenterol. **89**:1293.
9. Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser. 1990. Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. **265**:9494–9499.
10. Eaton, K. A., and S. Krakowka. 1994. Effect of gastric pH on urease-dependent colonization of gnotobiotic piglets by *Helicobacter pylori*. Infect. Immun. **62**:3604–3607.
11. Eaton, K. A., D. R. Morgan, C. L. Brooks, and S. Krakowka. 1991. Essential role of urease in pathogenesis of gastritis induced by *Helicobacter pylori* in gnotobiotic piglets. Infect. Immun. **59**:2470–2475.
12. Eaton, K. A., D. R. Morgan, and S. Krakowka. 1989. *Campylobacter pylori* virulence factors in gnotobiotic piglets. Infect. Immun. **57**:1119–1125.
13. Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne. 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. J. Bacteriol. **174**:4212–4217.
14. Fox, J. G., P. Correa, N. S. Taylor, A. Lee, G. Otto, J. C. Murphy, and R. Rose. 1990. *Helicobacter mustelae*-associated gastritis in ferrets. An animal model of *Helicobacter pylori* gastritis in humans. Gastroenterology **99**:352–361.
15. Fu, C., and R. J. Maier. 1994. Nucleotide sequences of two hydrogenase-related genes (*hypA* and *hypB*) from *Bradyrhizobium japonicum*, one of which (*hypB*) encodes an extremely histidine-rich region and guanine nucleotide-binding domains. Biochim. Biophys. Acta **1184**:135–138.
16. Grbac-Ivanovic, S., K. Antonijczuk, A. H. Varghese, M. C. Plowman, A. Antonijczuk, G. Korza, J. Ozols, and F. W. Sunderman, Jr. 1994. Lipovitellin 2beta is the 31 kD Ni^{2+} -binding protein (pNiXb) in *Xenopus* oocytes and embryos. Mol. Reprod. Dev. **38**:256–263.

17. Haas, R., T. F. Meyer, and J. P. M. van Putten. 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. *Mol. Microbiol.* **8**:753–760.
18. Hall, R., J. E. Hyde, M. Goman, D. L. Simmons, I. A. Hope, M. Mackay, J. Scaife, B. Merkli, R. Richle, and J. Stocker. 1984. Major surface antigen gene of a human malaria parasite cloned and expressed in bacteria. *Nature (London)* **311**:379–382.
19. Hausinger, R. P. 1987. Nickel utilization by microorganisms. *Microbiol. Rev.* **51**:22–42.
20. Hawtin, P. R., H. T. Delves, and D. G. Newell. 1991. The demonstration of nickel in the urease of *Helicobacter pylori* by atomic absorption spectroscopy. *FEMS Microbiol. Lett.* **77**:51–54.
21. Hu, L.-T., and H. L. T. Mobley. 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori*. *Infect. Immun.* **58**:992–998.
22. Kasprzak, K. S. 1991. The role of oxidative damage in metal carcinogenicity. *Chem. Res. Toxicol.* **4**:604–615.
23. Kelleher, J. E., and E. A. Raleigh. 1991. A novel activity in *Escherichia coli* K-12 that directs restriction of DNA modified at CG dinucleotides. *J. Bacteriol.* **173**:5220–5223.
24. Kilejian, A. 1974. A unique histidine-rich polypeptide from the malaria parasite, *Plasmodium lophurae*. *J. Biol. Chem.* **249**:4650–4655.
25. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**:105–132.
26. Lee, A., and S. L. Hazell. 1988. *Campylobacter pylori* in health and disease: an ecological perspective. *Microb. Ecol. Health Dis.* **1**:1–16.
27. Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger. 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protein that functions in urease metallocenter assembly. *Protein Sci.* **2**:1042–1052.
28. LeGendre, N., and P. Matsudaira. 1988. Direct protein microsequencing from Immobilon-P transfer membrane. *BioTechniques* **6**:154–159.
29. Lin, S. M., S. M. Hopfer, S. M. Brennan, and F. W. Sunderman, Jr. 1989. Protein blotting method for nickel-binding proteins. *Res. Commun. Chem. Pathol. Pharmacol.* **65**:275–288.
30. Makowski, G. S., S. M. Lin, S. M. Brennan, H. M. Smilowitz, S. M. Hopfer, and F. W. Sunderman, Jr. 1991. Detection of two Zn-finger proteins of *Xenopus laevis*, TFIIIA and p43, by probing Western blots of ovary cytosol with $^{65}\text{Zn}^{2+}$, $^{63}\text{Ni}^{2+}$, or $^{109}\text{Cd}^{2+}$. *Biol. Trace Elem. Res.* **29**:93–109.
31. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. Marshall, B. J., and J. R. Warren. 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i**:1311–1314.
33. Mobley, H. L. T., and R. P. Hausinger. 1989. Microbial ureases: significance, regulation, and molecular characterization. *Microbiol. Rev.* **53**:85–108.
34. Owen, R. J., S. R. Martin, and P. Borman. 1985. Rapid urea hydrolysis by gastric campylobacters. *Lancet* **i**:111.
35. Parsonnet, J., G. D. Friedman, D. P. Vandersteen, Y. Chang, J. H. Vogelman, N. Orentreich, and R. K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* **325**:1127–1131.
36. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. *Helicobacter pylori* infection and gastric lymphoma. *N. Engl. J. Med.* **330**:1267–1271.
37. Pérez-Pérez, G. I., C. B. Gower, and M. J. Blaser. 1994. Effects of cations on *Helicobacter pylori* urease activity, release, and stability. *Infect. Immun.* **62**:299–302.
38. Rabeneck, L., and D. F. Ransohoff. 1991. Is *Helicobacter pylori* a cause of duodenal ulcer? A methodologic critique of current evidence. *Am. J. Med.* **91**:566–572.
39. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
40. Senior, B. W., N. C. Bradford, and D. S. Simpson. 1980. The ureases of proteus strains in relation to virulence for the urinary tract. *J. Med. Microbiol.* **13**:507–512.
41. Silver, S., and M. Walderhaug. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**:195–228.
42. Spiegelhalter, C., B. Gerstenecker, A. Kersten, E. Schiltz, and M. Kist. 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. *Infect. Immun.* **61**:5315–5325.
43. Sriwanthana, B., M. D. Island, D. Maneval, and H. L. T. Mobley. 1994. Single-step purification of *Proteus mirabilis* urease accessory protein UreE, a protein with a naturally occurring histidine tail, by nickel chelate affinity chromatography. *J. Bacteriol.* **176**:6836–6841.
44. Suerbaum, S., J. M. Thiberge, I. Kansau, R. Ferrero, and A. Labigne. 1994. *Helicobacter pylori* hspA-hspB heat-shock gene cluster: nucleotide sequence, expression, putative function, and immunogenicity. *Mol. Microbiol.* **14**:959–974.
45. Tabata, M., and B. Sarkar. 1992. Specific nickel(II) transfer process between the native sequence peptide representing the nickel(II) transport site of human serum albumin and L-histidine. *J. Inorg. Biochem.* **45**:93–104.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
47. Walsh, C. T., and W. H. Orme-Johnson. 1987. Nickel enzymes. *Biochemistry* **26**:4901–4906.
48. Wulfig, C., J. Lombardero, and A. Pluckthun. 1994. An *Escherichia coli* protein consisting of a domain homologous to FK506-binding proteins (FKBP) and a new metal binding motif. *J. Biol. Chem.* **269**:2895–2901.