Properties of the P-Type ATPases Encoded by the \textit{copAP} Operons of \textit{Helicobacter pylori} and \textit{Helicobacter felis}

DENIS BAYLE,1 SABINE WANGLER,2 THOMAS WEITZENEGGER,3 JURGEN VOLZ,2 MICHAEL PRZYBYLSKI4 KLAUS P. SCHÄFER,2 GEORGE SACHS,1 AND KLAUS MELCHERS2*

University of California—Los Angeles and Wadsworth Veterans Affairs Medical Center, Los Angeles, California,1 and Department of Molecular Biology, Byk Gulden Pharmaceuticals,2 Gesellschaft für Analyse-Technik und Consulting GmbH,3 and Department of Chemistry, University of Konstanz,4 Konstanz, Germany

Received 5 August 1997/Accepted 3 November 1997

The \textit{cop} operons of \textit{Helicobacter pylori} and \textit{Helicobacter felis} were cloned by gene library screening. Both operons contain open reading frames for a P-type ion pump (CopA) with homology to Cd$^{2+}$ and Cu$^{2+}$ ATPases and a putative ion binding protein (CopP), the latter representing a CopZ homolog of the \textit{copYZAB} operon of \textit{Enterococcus hirae}. The predicted CopA ATPases contained an N-terminal GMXCCXC ion binding motif and a membrane-associated CPC sequence. A synthetic N-terminal peptide of the \textit{H. pylori} CopA ATPase bound to Cu$^{2+}$ specifically, and gene disruption mutagenesis of CopA resulted in an enhanced growth sensitivity of \textit{H. pylori} to Cu$^{2+}$ but not to other divalent cations. As determined experimentally, \textit{H. pylori} CopA copApump contains four pairs of transmembrane segments (H1 to H8), with the ATP binding and phosphorylation domains lying between H6 and H7, as found for another putative transition metal pump of \textit{H. pylori} (K. Melchers, T. Weitzenegger, A. Buhmann, W. Steinhilber, G. Sachs, and K. P. Schäfer, J. Biol. Chem. 271:446–457, 1996). The corresponding transmembrane segments of the \textit{H. felis} CopA pump were identified by hydrophobicity analysis and via sequence similarity. To define functional domains, similarly oriented regions of the two enzymes were examined for sequence identity. Regions with high degrees of identity included the N-terminal Cu$^{2+}$ binding domain, the regions of ATP binding and phosphorylation in the energy transduction domain, and a transport domain consisting of the last six transmembrane segments with conserved cysteine in H4, H6, and H7. The data suggest that \textit{H. pylori} and \textit{H. felis} employ conserved mechanisms of ATPase-dependent copper resistance.

\textit{Helicobacter pylori} is a human gastric pathogen associated with chronic gastritis, peptic ulcers, and gastric cancer (8, 16, 27, 30, 64). \textit{Helicobacter felis}, a related microorganism, was originally isolated from the stomach of a cat (42) but can also survive in the gastric environments of other mammalian species, such as mice. Mice infected with \textit{H. felis} are often used as a model to study \textit{H. pylori} colonization, pathogenicity, and eradication. Therefore, survival mechanisms of both \textit{H. pylori} and \textit{H. felis} are of interest. The specialized ecological niche of gastric \textit{Helicobacter} spp., the mammalian stomach, is dominated by the gastric acid produced by the H$^+$/K$^+$ ATPase of the parietal cells but also presents a highly variable cationic environment for these microorganisms. In bacteria, cation cell homeostasis as well as resistance to several transition metals is based on the action of both proton-cation antiporters and transition metal P-type ion pumps (36, 51). The ion pumps of \textit{H. pylori}, as well as those of other gastric \textit{Helicobacter} species, may participate in the survival mechanisms of the pathogenic bacterium.

Expression of some of these pumps is controlled either by a two-component system, consisting of a sensor kinase and a response regulator with affinity for specific DNA sequences, or by small cytoplasmic cation binding proteins acting as repressors or activators of gene expression. An example of the former is the high-affinity K$^+$ uptake ATPase encoded by the \textit{kdpABC} operon of \textit{Escherichia coli}. This operon was shown to be controlled by an adjacent operon containing two genes, \textit{kdpDE}, defining the sensor kinase and the corresponding response regulator of a two-component regulatory system (10, 25, 44, 63). While the \textit{KdpD} sensor kinase protein is membrane associated, the \textit{KdpE} regulator protein is cytoplasmic. The \textit{KdpDE} proteins regulate expression of the \textit{kdp} operon, apparently as a function of cellular turgor pressure (26, 59). In contrast, expression of the copper-transporting P-type ATPases in \textit{Enterococcus hirae}, \textit{CopA} and \textit{CopB} (39, 55), is thought to be controlled by two small cytoplasmic proteins, \textit{CopY} and \textit{CopZ} (38). The \textit{copYZ} genes, which encode small transition metal binding proteins, precede the structural P-type ATPase genes \textit{A} and \textit{B} as part of the enterococcal \textit{copYZAB} operon. Their protein products were postulated to act as repressors (\textit{CopY}) or activators (\textit{CopZ}) regulating gene transcription of the \textit{cop} operon depending on the availability of Cu$^{2+}$ (38). The \textit{CopAB} P-type ATPases of \textit{Enterococcus hirae} belong to a family of transition metal ATPases containing N-terminal ion binding motifs and a membrane-associated CPX sequence, both suggested to play a role in ion binding and/or ion transport (5, 29, 51, 52, 57).

Of special interest is the membrane topology of P-type ATPases, which provides a structural basis for the ion transport pathway through the membrane. Hydrophathy profiles, as well as other computer-aided methods for detection of amphipathic helices in membrane proteins, have been misleading, and therefore the determination of topology needs experimental evaluation (7, 50). The first bacterial P-type ATPase to have its transmembrane (TM) segments defined experimentally was the Mg$^{2+}$ ATPase of \textit{Salmonella typhimurium} (54). This membrane pump was shown to contain 10 membrane-spanning helices, with the large cytoplasmic ATP binding and phosphor-
ylation loop being between H4 and H5. This ATPase therefore resembles the eukaryotic alkali metal P-type pumps which transport alkali cations as well as Mg$^{2+}$ and Ca$^{2+}$, the latter also being relatively small cations (21). The amino acid sequences of transition metal ATPases exhibit a different hydrophobic profile, with two additional hydrophobic domains in the N-terminal region and four fewer in the C-terminal region. The core structure of six TM helices is likely to be similar (28). The first transition metal ATPase investigated experimentally for the number and orientation of TM segments was an H. pylori P-type pump (31) most closely related to the Cd$^{2+}$ ATPase of Staphylococcus aureus, CadA, the latter previously being thought to contain only six TM segments (52, 53). However, the H. pylori ATPase was shown to contain a membrane domain of eight TM helices comprising the additional N-terminal pair of membrane spans followed by the core structure of six membrane spanning segments with only one pair of C-terminal TM segments (31).

Here we describe the isolation of the copAP operons of H. pylori 69A and H. felis ATCC 49179. They both carry two genes, one encoding another member of the bacterial transition metal ion ATPase family, CopA, and the other encoding a putative cation binding regulatory peptide of 66 amino acid residues, CopP. The small CopP peptide is homologous to CopZ, encoded by the E. hirae copZ operon (38). In a previous study using a truncated variant of the ATPase cloned from H. pylori UA802, it was claimed that the pump is a Cu$^{2+}$ export ATPase (13). In this study, the function of the H. pylori 69A-derived ATPase was analyzed by knockout mutagenesis of H. pylori copA, and we also investigated the N-terminal ion binding properties of the putative Cu$^{2+}$ ATPase by ion affinity chromatography and electrospray ionization mass spectrometry (ESI-MS). Compared to the other P-type pump cloned from H. pylori 69A (31), the DNA-derived amino acid sequence of the CopA P-type pumps discussed here contains additional hydrophobic segments, and therefore a topological analysis was performed on the H. pylori CopA ATPase. The results obtained by experimental identification of membrane-inserted segments and the homology analysis of the CopA pumps cloned from the two related microorganisms, H. pylori and H. felis, allowed detection of domains of high amino acid sequence identity that have perhaps been conserved to transport copper across the cytoplasmic membrane in these gastric bacteria.

**MATERIALS AND METHODS**

**Bacterial strains.** *E. coli* HB101 containing the H. pylori gene library was a gift of Rainer Haas (Tübingen, Germany). Replication of random pRH948-derived DNA fragments in vector pTZ18R for subsequent DNA sequencing was performed in *E. coli* K12, a gift from I. Rasched (Konstanz, Germany). *E. coli* MM294, supplied by the American Type Culture Collection (ATCC 32625), was used for plasmid cloning experiments. *E. coli* XLI-Blue MRF$^+$ and SOLR, both obtained from Stratagene, were used for amplification of H. felis genomic library in the Lambda ZAP II vector. *E. coli* strains used were grown as recommended by the supplier of the strains (Stratagene). *H. pylori* strains were grown in brain heart infusion (Difco) medium (BHIB) containing 5% horse serum and 2% serum (DIFCO) in a CO$_2$ incubator (10% CO$_2$) at 37°C in 10-ml cell culture flasks. Growth was monitored by determination of the optical density at 578 nm of aliquots of the bacterial cultures at various time points. *H. felis* cells were grown in Columbia EB broth (Difco) supplemented with 6% horse serum in GasPak jars under microaerophilic conditions (Anaerocult C; Merck) at 37°C in a shaker incubator.

Selection of DNA oligonucleotide sequences for detection of P-type ATPases. All known P-type ATPases from eukaryotic and bacterial cells contain a highly conserved DKTG(T/L)T phosphorylation consensus sequence (11, 43). This sequence can be encoded by a pool of 2,304 different 20-base oligonucleotides (maxipool) covering all possible sequences of the phosphorylation consensus sequence as well as the AC of the threonine codon. This pool of oligonucleotides was divided into five chemically synthesized oligonucleotide subpopulations (subpools I-405 to I-409). The DNA nucleotide sequences of the synthesized subpools consisted of G(A/C)T (AC/GT) with a 3′ extension of AT(AC) (AC for I-405), AT(CA) (AC for I-406), TT(AG) AC (I-407), CT(TG) AC (for I-408), or CT(AG) AC (for I-409). The variable nucleotides are in parentheses. The DNA primers (MWG Biotech, Ebersberg, Germany) were labeled by using a digoxigenin labeling kit (Boehringer Mannheim). Each of the primers listed above was employed for genomic Southern blot analysis (58). Subsequently, primer I-408 was selected for Southern blot screening of the H. pylori 69A library in plasmid pH160 and plaque screening of membranes filters obtained from the H. felis gene library in the Lambda ZAP II phase. I-408 was also used for initial DNA sequencing to verify that positive plasmids contained the DKTG(T/L)T target sequence.

**Preparation of DNA library.** Genomic DNA of H. pylori or H. felis was prepared by standard protocols as described previously (1, 48). Plasmid DNA was isolated by anion-exchange chromatography (Qiagen).

**Southern blot analysis of H. pylori genomic DNA with various DNA oligonucleotide probes.** The DNA of H. pylori was digested with the restriction enzyme Sau3AI and transferred to nitrocellulose. The digested DNA was separated by agarose gel electrophoresis, denatured, and blotted onto nylon membranes. Each of the Southern blots contained the restriction endonuclease-digested H. pylori DNA was hybridized with one of the DIG-labeled DNA oligonucleotides and washed in accordance with a Boehringer Mannheim protocol. Hybridization was performed for >6 h in a solution containing 5× standard saline citrate (SSC) buffer (2 M NaCl plus 0.15 M sodium citrate, 1× blocking agent, 0.2% (wt/vol) sodium dodecyl sulfate (SDS), and 0.1% (wt/vol) N-laurylsarcosine at 45°C. Hybridized blots were washed in 5× SSC buffer–0.1% (wt/vol) SDS at 45°C. Positive restriction fragments were detected by chemiluminescence (Boehringer Mannheim).

**H. pylori 69A gene library.** The DNA library was constructed by Rainer Haas. It contains genomic DNA fragments of H. pylori 69A. The DNA fragments generated by partial Sau3AI digestion were cloned into the BlII restriction site of the plasmid pH160, also referred to as the pM11 vector (18). The library was replicated in E. coli HB101. G418 resistant clones were screened by Southern blot analysis of genomic DNA isolated from this library. A positive clone was selected, and the DNA was purified by alkaline lysis and ethanol precipitation.

**Screening of gene libraries for detection of P-type ATPases.** (i) Southern blot screening and isolation of pH vectors containing putative P-type ATPase genes of *H. pylori*. Since the phosphorylation consensus target site selected for screening is present in all known P-type pumps, the *H. pylori* gene library was screened by hybridization to aliquots of isolated plasmid DNA mixtures to avoid contaminating signals from the *E. coli* genome. For preparation of distinct plasmid DNA mixtures, an aliquot of the library, 1.4 × 10$^9$ CFU, was diluted in LB broth medium supplemented with 50 μg of tetracycline per ml. The bacterial clones were used for inoculation of 20 cultures for subsequent mixed-plasmid preparations. Inoculated vials, each containing approximately 70 different plasmids of the library, were incubated overnight in a shaker incubator at 37°C. Mixed plasmid DNA was purified by ion-exchange chromatography (Qiagen). In addition, glycerol was added to an aliquot of each of the mixed bacterial suspensions, which were then stored at −70°C. The isolated plasmid DNA mixtures were subjected to digestion with restriction endonucleases EcoRI and XhoI. DNA fragments were separated on 1% (wt/vol) agarose gels and blotted onto nylon membranes (38). The membranes containing restriction enzyme-digested DNA of the mixed-plasmid preparations were hybridized with DIG-labeled DNA oligonucleotide I-408 according to the protocol of Boehringer Mannheim as described above for genomic Southern blot analysis. Positive plasmid mixtures were detected by chemiluminescence (Boehringer Mannheim). Aliquots taken from corresponding glycerol stocks were plated out on LB agar plates containing 50 μg of tetracycline per ml, and 60 colonies were selected for preparation of clonal plasmid DNA using an anion-exchange column (Qiagen) and digestion of the first six amino acid ends of the clones hybridized in the mixed plasmid preparation. Plasmid DNAs were analyzed for sequences homologous to primer I-408 by another cycle of Southern blot analysis as described above. Three distinct positive DNA clones containing H. pylori 69A DNA fragments flanked by EcoRI and XhoI restriction sites were chosen. Southern blot analysis of the cloning vector were isolated, namely pRH154, pRH539, and pRH498.

Downloaded from http://jbp.asm.org/ on August 28, 2017 by guest
plasmids were subjected to a cycle of DNA sequencing for detection of the DNA target sequence corresponding to the phosphorylation consensus sequence.

(ii) Plaque filter hybridization and in vivo excision of pHF vectors containing putative P-type ATPase genes of \textit{H. felis}. The \textit{H. felis} gene library was screened by conventional plaque filter hybridization. Nylon membranes (Amersham) containing phage DNA from 2 \times 10^6 plaques were prepared by standard protocols (48). DNA-containing membranes were hybridized with primer I-408 under the same conditions used for Southern blot screening of the \textit{H. pylori} \textit{69A} gene library. From positive Lambda ZAP II phage clones, 10 \muL of primer I-408 was cut with \textit{EcoRI} and \textit{BamHI} restriction enzymes. The DNA sequences of the first pair of primers were ACCGAGTTGAATTCATG and a \textit{BamHI} recognition site, for I-500 and CTGCAACTCAAGC for I-480, including the ATG/Met-135 codon of \textit{copA} putative P-type ATPase genes of \textit{H. pylori}.

PCR products were cut with \textit{EcoRI} and \textit{BamHI} restriction enzymes and cloned into \textit{pUC} 19. BHI broth medium was washed twice at 4°C in 10% (vol/vol) vol/vol) and resuspended to 10% (vol/vol) glycerol at about 10^8/mL. PY-123 DNA (2.5 mg) was added to 200 \muL of \textit{H. pylori} cell suspension. The mixture, after incubation on ice for 10 min, was transferred into a prechilled 0.2-cm cuvette (Bio-Rad) and subjected to single-pulse electrophoresis in a Bio-Rad Gene Pulser. Cells were subjected to BHI agar plates for 24 h incubation under microaerophilic conditions at 37°C. The cells were subsequently transferred to BHI agar plates containing 10 \muL of kanamycin sulfate per ml and incubated for 5 days as described above. Transformants obtained were grown in 10 ml of BHI-yeast extract-horse serum medium supplemented with 10 \muL of kanamycin sulfate per ml.

Verification of \textit{copA} knockout mutants of \textit{H. pylori} by PCR. Genomic DNA of \textit{H. pylori} mutants and parental \textit{H. pylori} were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Effect of metal ions on growth of \textit{H. pylori}. To determine the MICs of various divalent cations for \textit{H. pylori} wild-type strain ATCC 49505 or its \textit{copA}-deficient derivative, cells were incubated in BHI broth medium in the absence or presence of various concentrations of Ca^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, or Mg^{2+} as the Cl^- salt. Growth of the bacterial cultures was measured by measurement of optical density (578 nm) at various time points. Concentrations employed were 50 \muM, 100 \muM, 250 \muM, 500 \muM, and 1 mM for all cations listed above except for Mg^{2+}. Additional concentrations used were 2.5, 7.5, 15, and 25 \muM for CuCl_2, and 2 mM and 4 mM and 7 mM for NiCl_2. Concentrations used to determine MICs of MgCl_2 were 5, 25, 50, and 75 mM.

Synthesis of the N-terminal peptide of \textit{H. pylori} CopA ATPase (amino acid residues 1 to 52). A 25-\muL volume of the ATPase peptide from the T-nmethylmethionyl to leucine-52 was synthesized by solid-phase peptide synthesis with an Abimed EPS221 automated peptide synthesizer (Abimed, Langefeld, Germany), using the fluorenylmethoxycarbonyl (Fmoc) protection strategy (6, 12, 32). The N\textsuperscript{t}-protected leucine residue was carboxy-terminally linked by 4-hydroxybenzylphenoxyacetic acid to a graft polymer of polyethylene glycol onto a polystyrene support (5) (Novy\textsuperscript{a} TGA resin; Novabiochem, Bad Soden, Germany). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) activator and N\textsuperscript{t}-protected amino acids were also purchased from Novabiochem (7a). Reagents and solvents were obtained from Aldrich (N-methylmorpholine), Merck (dimethylformamide), and Fluka (pipерidine, tri- methylmorpholine, and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) (Qiagen, Hilden, Germany). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) activator and N\textsuperscript{t}-protected amino acids were also purchased from Novabiochem (7a). Reagents and solvents were obtained from Aldrich (N-methylmorpholine), Merck (dimethylformamide), and Fluka (pipерidine, tri- methylmorpholine, and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) (Qiagen, Hilden, Germany). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) activator and N\textsuperscript{t}-protected amino acids were also purchased from Novabiochem (7a). Reagents and solvents were obtained from Aldrich (N-methylmorpholine), Merck (dimethylformamide), and Fluka (pipерidine, tri- methylmorpholine, and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) (Qiagen, Hilden, Germany). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) activator and N\textsuperscript{t}-protected amino acids were also purchased from Novabiochem (7a). Reagents and solvents were obtained from Aldrich (N-methylmorpholine), Merck (dimethylformamide), and Fluka (pipерidine, tri- methylmorpholine, and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) (Qiagen, Hilden, Germany). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) activator and N\textsuperscript{t}-protected amino acids were also purchased from Novabiochem (7a). Reagents and solvents were obtained from Aldrich (N-methylmorpholine), Merck (dimethylformamide), and Fluka (pipерidine, tri- methylmorpholine, and benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) (Qiagen, Hilden, Germany). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) activator and N\textsuperscript{t}-protected amino acids were also purchased from Novabiochem (7a). Reagents and solvents were obtained from Aldrich (N-methylmorpholine), Merck (dimethylformamide), and Fluka (pipercyclase treatment with trifluoroacetic acid for 2 h at 20°C, using a triethylsilyl scavenger. The crude peptide was isolated by precipitation with p-butylnethyl ether (Fluka) and semipreparative high-performance liquid chromatography (Waters Bondapak C\textsubscript{18} column) with a linear gradient of water and acetonitrile. The high-performance liquid chromatography-purified peptide was subjected to matrix-assisted laser desorption mass spectrometry (20, 45) and showed an average molecular mass of 5,851 Da, which was in good agreement with the molecular mass of 5,850.7 Da calculated from the amino acid sequence.

Metal ion binding by the N-terminal peptide of a synthetic ATPase. N\textsuperscript{t}-affinity chromatography (Qiagen, Hilden, Germany) was used to study the ability of the first N-terminal 52 amino acids of the ATPase peptide (P95-030) to bind metal ions. To exchange the N\textsuperscript{t}-ions for other divalent cations, 1 ml of N\textsuperscript{t}+-agarose suspension (equivalent to 500 \muL of gel bed) was loaded onto a column (3-cm Econo; Bio-Rad), which was subsequently washed with 2 ml of H\textsubscript{2}O followed by 5 ml of 100 mM ethylenediaminetetraacetic acid (EDTA) to remove the N\textsuperscript{t}+-ions and then equilibrated with 2 ml of H\textsubscript{2}O. The binding of the ions to be tested to the agarose matrix was carried out by incubating the agarose matrix with 2 ml of a 100 mM solution of either NiCl\textsubscript{2}, CuSO\textsubscript{4}, CoCl\textsubscript{2}, ZnCl\textsubscript{2}, CdCl\textsubscript{2}, or MgCl\textsubscript{2} for 2 h at 20°C. After transfer to an Econo 40-mL gel bed column, the column was washed first with 2 times 2 ml of H\textsubscript{2}O and then with binding buffer, pH 7.8 (50 mM
were prepared in 5 mM ammonium acetate in water-methanol (9:1) at pH 4. A 0.025% Serva Blue G in 10% acetic acid for 1 h and destained in 10% acetic acid. Mental conditions were as described previously (44). Peptide solutions (50 µg) were heated to 95°C for 3 min and then transferred to a 1.5-ml reaction tube, and the mixture was incubated for 1 h at room temperature in a 1-ml fraction obtained by elution of the affinity agarose column was dried in a vacuum concentrator and resuspended in 40 µl of SDS-Tricine-polyacrylamide gel electrophoresis (PAGE) sample buffer. The precipitates were separated by SDS-PAGE on a 1.5-ml reaction tube, and the mixture was incubated for 1 h at a flow rate of 3 µl/min. Sample delivery to the electrospray needle tip was performed with a Harvard-44 microinfusion pump (Harvard Apparatus, South Natick, Mass.) and a 1.5-ml Mobitec column tube. Bound peptide was eluted with 3 ml of binding buffer, and the mixture was incubated for 1 h at room temperature on a roller incubator. After this binding step, the supernatant was removed, and 30 µl was dried in a vacuum concentrator and resuspended in 40 µl of SDS-Tricine-polyacrylamide gel electrophoresis (PAGE) sample buffer. The matrix was washed once with 3 volumes of binding buffer and transferred to a 1.5-ml Mobitec column tube. Bound peptide was eluted with 3 ml of binding buffer adjusted to pH 4 with 1 N HCl and collected in two fractions of 1 and 2 ml.

PAGE of ATPase peptide. The peptide of 52 amino acids (M1-L52) contained in the 1-ml fraction obtained by elution of the affinity agarose column was precipitated with 20% trichloroacetic acid and resuspended in 40 µl of SDS-Tricine-polyacrylamide gel electrophoresis (PAGE) sample buffer. The precipitates were separated by SDS-PAGE on a 15% polyacrylamide gel with Tricine buffer (49). The gels were stained with 0.025% Serva Blue G in 10% acetic acid for 1 h and destained in 10% acetic acid.

ESI-MS of the ATPase peptide. ESI spectra of the N-terminal peptide of the synthetic ATPase were recorded on a Vestec 201 A single-quadrupole mass spectrometer (Vestec Corp., Houston, Tex.) through a fused-silica capillary tube at a flow rate of 3 µl/min.

RESULTS

Genomic Southern blot analyses. A DNA oligonucleotide mixture of 16 distinct 20-mer DNA molecules out of the 2,304 possible sequences encoding the DKTGT(I/L)T phosphorylation consensus sequence had been used previously to isolate an H. pylori P-type ATPase (31). In this study, five DNA oligonucleotide mixtures covering all possible DNA sequences of the phosphorylation target sequence, I-405 to I-409, were used for Southern blot analyses of H. pylori chromosomal DNA. Each of the synthesized DNA oligonucleotide mixtures, consisting of similar DNA sequences, was hybridized to membranes containing HindIII-restricted genomic DNA. In all cases, a pattern of up to five major hybridization bands was found, indicating a high degree of similarity but nonidentity (Fig. 1). This shows that even the phosphorylation site-specific DNA oligonucleotides of extended complexity are able to detect putative P-type...
ATPase DNA clones derived from _H. pylori_ DNA. Since primer I-408 detected all of the major bands, this primer was selected for use in screening of the _H. pylori_ 69A gene library. The same primer was used for screening of an _H. felis_ gene library.

Isolation of _Helicobacter copA_ operons. Since _E. coli_ DNA was also positive with each of the five DNA oligonucleotide probes (data not shown), the _H. pylori_ 69A library was screened by subjecting mixed-plasmid preparations to Southern blot hybridization technique. Using probe I-408, plasmid pRH948 as well as two additional DNA clones, pRH514 and pRH539, were isolated from the _H. pylori_ genomic DNA library present in pRH160 in _E. coli_ HB101. The lengths of the inserted _H. pylori_ DNA sequences of the plasmids were determined by restriction analysis to be within the range of 3 to 5 kbp (data not shown).

DNA sequencing revealed that pRH948 contained a novel P-type ATPase gene, whereas the DNA sequence of pRH514 showed identity with the ion pump isolated previously (31), and pRH539 was not susceptible to the DNA sequencing procedure using primer I-408. Hence, vector pRH948 was selected for sequencing of the complete _H. pylori_ DNA fragment by the direct blotting procedure as described in Materials and Methods.

Primer I-408 was also used to screen an _H. felis_ gene library cloned in the _λ_ ZAP II vector. Screening led to the isolation of 10 positive clones, pHF1 to pHF10. Partial sequencing showed that four of the clones isolated (pHF1, -2, -6, and -8) were identical, containing DNA sequences encoding a P-type ATPase. pHF8 was selected for DNA sequencing.

DNA sequence analysis of _Helicobacter copA_ operons and flanking DNA. DNA sequencing showed that the _H. pylori_ DNA sequence in pRH948 was 4,472 bp in length. About 2.3 kbp of the pRH948 3' DNA sequence overlaps with a previously published sequence predicted to contain the copA operon of _H. pylori_ UA802 (13). Since this published sequence encodes a membrane pump lacking an N-terminal ion binding domain as well as the first pair of _TM_ segments, it was assumed that this earlier-cloned version of the _H. pylori_ CopA ion pump was N-terminally truncated (31). The DNA sequence inserted in pRH948 contained an additional 2.2 kbp of the nucleotide sequence region upstream of the DNA reported previously (13) before enclosing the N-terminal sequences not present in the first version of CopA. The missing sequences of the _H. pylori_ UA802-encoded version of the CopA ion pump have been published recently (14). Sequence identity between the _H. pylori_ 69A-derived DNA and the corresponding DNA region of _H. pylori_ UA802 was found to be about 94%. The copAP locus, with a degree of DNA sequence identity similar to that of the former operons, is also present in _H. pylori_ 26695 (60).

The sequence of the pRH948 DNA strand spanning the distance between the EcoRI cloning site and the XhoI recognition site of the vector backbone was found to predict five open reading frames (ORFs), whereas no ORF of any significant length was found on the reverse strand. The two ORFs located at the terminal regions of the inserted DNA fragment, ORF1 and ORF5, were interrupted by the cloning sites and therefore were incomplete. ORF1 encoding an N-terminally truncated version of the _H. pylori_ FtsH protein, is also present upstream of the copAP operon in _H. pylori_ UA802 (15). The next coding region (ORF2), which was separated from the preceding ftsH sequence by a putative transcriptional termination signal, predicted a protein of 237 amino acids. An N-terminal overlap of 173 amino acid residues of this amino acid sequence showed the closest identity to the phosphatidylethanolamine synthase of _Bacillus subtilis_, a protein of 177 amino acids (40).

ORF2 was followed by the largest ORF of pRH948, ORF3, which contains the P-type ATPase target sequence used for screening. The P-type gene of ORF3 (copA) is immediately followed by a small ORF4 encoding a peptide of 66 amino acids (CopP). The 3'-localized ORF of pRH948 (ORF5) is interrupted by vector sequences immediately downstream. The predicted N-terminal peptide of 27 amino acids, which is not identical to the gene product predicted by the corresponding region in _H. pylori_ UA802 (13), is of unknown homology. In Fig. 2, a structural map of the pRH948 insertional DNA is given, showing the organization of the _H. pylori_ copA operon and of the ORFs flanking the operon. Putative transcription termination sequences are present in the DNA sequence downstream of the truncated ftsH region and also downstream of _copP_, separating ORF4 and ORF5. An AGGA Shine-Dalgarno consensus sequence is located 7 bases upstream of the _copP_ gene, whereas _copA_, in the corresponding region, contained a DNA sequence with little similarity to the Shine-Dalgarno consensus motif (data not shown).

DNA sequencing of the _H. felis_ 5,909 kbp insertion of pHF8 showed that this related organism also contained a cop operon consisting of genes encoding a P-type ATPase (CopA) and a small peptide with possible transition metal ion binding prop-

FIG. 1. Fluorogram of a Southern blot of chromosomal _H. pylori_ 69A DNA hybridized with various DNA oligonucleotides targeted to the phosphorylation signature sequences of _P_-type ATPases. The membranes containing HindIII-restricted DNA were hybridized with DIG-labeled primers I-405 (lane 1), I-406 (lane 2), I-407 (lane 3), I-408 (lane 4), and I-409 (lane 5), as described in Materials and Methods. Positive restriction fragments were detected by chemiluminescence. Molecular sizes are given in kilobase pairs.
FIG. 3. Comparison of the amino acid sequences of CopZ from Enterococcus hirae and the CopP protein from Helicobacter pylori (Hp) strains and Helicobacter felis (Hf). The CopP sequences of 66 amino acid residues encoded by plasmids pRH948 (Hp, strain 69A) and pRH948 (this study) and pRH948 (Hp, strain UA802) (13) or detected by H. pylori genome sequencing (Hp pylori 26695) (60) have an overall identity of >95%. The CopP peptide predicted from the small ORF of the copAP DNA fragment of H. felis, also consisting of 66 amino acids, showed a lower degree of identity with the H. pylori peptides (about 60%), but a stretch of 10 identical amino acids was observed just after the CXXC motif. The degree of amino acid sequence identity of CopP peptides and E. hirae CopZ (69 amino acid residues) is still between 40 and 50%.

Properties of CopP amino acid sequences. The CopP gene products of H. pylori 69A and H. felis both consist of 66 amino acids and have about 60% identity (Fig. 3). They both contain a CXXC-type transition metal binding motif, suggesting that this protein may act as a transition metal binding protein. This protein was also predicted from the copAP DNA region of H. pylori 26695 and UA802 (13-15, 60). The encoded proteins both contain a DK-ATP binding motif, as found in other P-type ion pumps (11, 43). There are also consensus sequences characteristic of transition metal P-type ATPases, such as an N-terminal GMX-CXXC sequence motif and a CPC box in the membrane domain (52, 57). The amino acid sequence of the H. pylori 69A CopP protein exhibited 95% identity with the CopA protein products cloned from H. pylori 26695 (60) and H. pylori UA802, the latter containing a CPS motif instead of the CPC found in the H. pylori 69A and 26695 CopA sequences (13).

FIG. 4. Comparison of the CopA amino acid sequences of H. pylori (Hp) and H. felis (Hf). The CopA amino acid sequences are 741 (Hp) and 732 (Hf) amino acids in length, as shown in Fig. 4. The encoded proteins both contain a DK-TGTTLT phosphorylation signature sequence and a GDGVDN ATP binding motif, as found in other P-type ion pumps (11, 43). There are also consensus sequences characteristic of transition metal P-type ATPases, such as an N-terminal GMX-CXXC sequence motif and a CPC box in the membrane domain (52, 57). The amino acid sequence of the H. pylori 69A CopP protein exhibited 95% identity with the CopA protein products cloned from H. pylori 26695 (60) and H. pylori UA802, the latter containing a CPS motif instead of the CPC found in the H. pylori 69A and 26695 CopA sequences (13).

Of the proteins currently available in the database, the H. pylori 69A and H. felis CopA exhibited the highest degree of sequence homology of the proteins currently available in the database, to another ATPase of H. pylori cloned in our laboratory (31) and to the various bacterial and eukaryotic Cu²⁺ and Cd²⁺ ATPases. Prominent members of this family are CopA and CopB of Enterococcus hirae (39, 55); the Cd²⁺ ATPase of Staphylococcus aureus; CadA (37), a putative Synecochoccus Cu²⁺ ATPase (19); and the human Menkes and Wilson gene products (5, 62). From the degree of amino acid sequence similarity and the occurrence of both GMXXC and CPC motifs, it was concluded that the cloned pump is a P-type ATPase.

Properties of CopA amino acid sequences. The CopA gene products of H. pylori 69A and H. felis both consist of 66 amino acids and have about 60% identity (Fig. 3). They both contain a CXXC-type transition metal binding motif, suggesting that this protein may act as a transition metal binding protein. This protein was also predicted from the copAP loci of H. pylori UA802 and H. pylori 26695 (13, 60). The CopA peptides of H. pylori and H. felis were found to exhibit homology with other ion binding proteins, such as the periplasmic mercury binding protein MerP of Serratia marcescens, MerP of Shigella flexneri, and especially the CopZ protein of Enterococcus hirae (33, 35, 39). Figure 3 displays a sequence alignment of enterococcal CopZ (35), CopP from H. felis (this study), and the CopA amino acid sequence variants cloned from H. pylori 69A (this study) and other H. pylori strains (13, 60). They all contain a CXXC motif in amino acid positions 12 to 15.

Properties of CopA amino acid sequences. The P-type pumps predicted by the Helicobacter cop operons were 741 (CopA, H. pylori) or 732 (CopA, H. felis) amino acids in length, as shown in Fig. 4. The encoded proteins both contain a DK-TGTTLT phosphorylation signature sequence and a GDGVDN ATP binding motif, as found in other P-type ion pumps (11, 43). There are also consensus sequences characteristic of transition metal P-type ATPases, such as an N-terminal GMX-CXXC sequence motif and a CPC box in the membrane domain (52, 57). The amino acid sequence of the H. pylori 69A CopA protein exhibited 95% identity with the CopA protein products cloned from H. pylori 26695 (60) and H. pylori UA802, the latter containing a CPS motif instead of the CPC found in the H. pylori 69A and 26695 CopA sequences (13).
The peptide was preincubated with CuCl₂ or NiCl₂. When three, four, and fivefold positively charged ions of the peptide, determined by metal ion affinity chromatography. A 100-μg portion of peptide P95-030 was bound to a divalent-cation column as described in Materials and Methods. Bound peptide was eluted and separated by SDS-PAGE, using 15% acrylamide and Tricine buffer under nonreducing conditions, and stained with Serva Blue G. Lane 1, 5 μg of peptide P95-030 (control); lanes 3 to 8, peptide eluted from the matrix after binding to divalent ions and contained in 1 ml of the 3-ml elution volume. The column matrix was equilibrated with CuCl₂ (lane 3), NiCl₂ (lane 4), CoCl₂ (lane 5), CdCl₂ (lane 6), MgCl₂ (lane 7), or ZnCl₂ (lane 8). The peptide was able to form dimers, presumably due to the formation of intermolecular Cys-Cys bonds (lanes 1, 3, and 8).

FIG. 5. Ion binding affinity of a synthetic ATPase peptide (P95-030) as determined by metal ion affinity chromatography. A 100-μg portion of peptide P95-030 was bound to a divalent-cation column as described in Materials and Methods. Bound peptide was eluted and separated by SDS-PAGE using 15% acrylamide and Tricine buffer under nonreducing conditions, and stained with Serva Blue G. Lane 1, 5 μg of peptide P95-030 (control); lanes 3 to 8, peptide eluted from the matrix after binding to divalent ions and contained in 1 ml of the 3-ml elution volume. The column matrix was equilibrated with CuCl₂ (lane 3), NiCl₂ (lane 4), CoCl₂ (lane 5), CdCl₂ (lane 6), MgCl₂ (lane 7), or ZnCl₂ (lane 8). The peptide was able to form dimers, presumably due to the formation of intermolecular Cys-Cys bonds (lanes 1, 3, and 8).

The function of the cloned CopA ATPase was determined by gene disruption mutagenesis of the ATPase gene contained in pRH498 and subsequent generation of H. pylori copA knockout mutants by homologous recombination, as described in Materials and Methods. This methodology is not yet available for H. felis. The wild-type strain subjected to copA mutagenesis was H. pylori ATCC 49503. In the mutant obtained, the copA ATPase gene lacked an entire segment of the pump, from amino acid 301 to 496, which was exchanged for a kanamycin resistance cassette, resulting in a kanamycin resistance phenotype in the recipients of the PY-123 construct. Neither the growth kinetics of the ATPase-deficient mutants nor their urease activities were different from that of the parental strain, H. pylori ATCC 49503, showing that this pump is not necessary for growth in vitro (data not shown). However, the enzyme might still be essential for survival in the stomach.

The mutant was susceptible to the same concentrations of Ni²⁺, Zn²⁺, Co²⁺, and Mg²⁺ as the wild-type strain but had a different susceptibility to Cu²⁺. The wild-type strain had a Cu²⁺ MIC of 50 μM, whereas the mutant had a Cu²⁺ MIC of 7.5 μM, suggesting that the ATPase can function as a Cu²⁺ export pump (Table 2). A corresponding change in copper sensitivity was obtained when the pRH498-encoded CPC-type ATPase was inactivated in the genome of H. pylori 69A by transposon shuttle mutagenesis (data not shown). Since a change in Cu²⁺ sensitivity was also found in H. pylori UA802 copA knockout mutants (13), it is evident that both of the natural CopA sequence variants, the CPC-type pump predicted by pRH498 and the CPS-containing sequence predicted from the copA gene cloned from H. pylori UA802, are able to export Cu²⁺.

Metal binding characteristics of the N-terminal region of the ATPase. When the synthetic peptide representing the 52 N-terminal amino acids of H. pylori CopA containing the GMXCCXXC ion binding motif was adsorbed to affinity agarose equilibrated with Ni²⁺, Co²⁺, Cd²⁺, Mg²⁺, Zn²⁺, or Cu²⁺, the peptide displayed binding mainly to Cu²⁺. A very weak binding reaction was observed with Zn²⁺. These data are shown in Fig. 5.

The spectra obtained by subjecting the N-terminal ATPase peptide to ESI-MS are depicted in Fig. 6. ESI-MS detected the three, four, and fivefold positively charged ions of the peptide. The peptide was preincubated with CuCl₂ or NiCl₂. When preincubated in the presence of CuCl₂, adducts of the peptide carrying up to three copper atoms were observed, as demonstrated in Fig. 6B. Copper ion binding of the peptide is in agreement with the data obtained by ion affinity chromatography.

The mutant was susceptible to the same concentrations of Ni²⁺, Zn²⁺, Co²⁺, and Mg²⁺ as the wild-type strain but had a different susceptibility to Cu²⁺. The wild-type strain had a Cu²⁺ MIC of 50 μM, whereas the mutant had a Cu²⁺ MIC of 7.5 μM, suggesting that the ATPase can function as a Cu²⁺ export pump (Table 2). A corresponding change in copper sensitivity was obtained when the pRH498-encoded CPC-type ATPase was inactivated in the genome of H. pylori 69A by transposon shuttle mutagenesis (data not shown). Since a change in Cu²⁺ sensitivity was also found in H. pylori UA802 copA knockout mutants (13), it is evident that both of the natural CopA sequence variants, the CPC-type pump predicted by pRH498 and the CPS-containing sequence predicted from the copA gene cloned from H. pylori UA802, are able to export Cu²⁺.

TABLE 2. MICs of divalent cations for H. pylori

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>MIC of divalent cation for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H. pylori ATCC 49503</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>50 μM</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>500 μM</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>4 mM</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>250 μM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>50 mM</td>
</tr>
</tbody>
</table>

* To determine the MICs of various divalent cations for H. pylori, cells of the wild-type strain ATCC 49503 or its CopA-deficient derivative were incubated in BHI broth medium in the absence or presence of various concentrations of CuCl₂, ZnCl₂, CoCl₂, or NiCl₂. The growth MICs for the cations are shown.

FIG. 6. Electrospray mass spectra of synthetic ATPase peptide (amino acids 1 to 52) in the absence and presence of Cu²⁺ and Ni²⁺. (A) ESI spectra of unmodified Cop ATPase peptide. Three, four, and fivefold positively charged ions of the peptide were detected. (B) After preincubation with CuCl₂, 4.5-fold positively charged adducts of copper with the peptide were observed. (C) When the peptide was preincubated with NiCl₂, complexes of the peptide with nickel seemed to be detectable also, as indicated by the very faint peaks immediately following the [M + 4H]⁺ signal. Binding to Ni²⁺, therefore, was much less significant than binding to Cu²⁺. M, peptide molecule; H, proton; m, mass; z, charge of molecule.
sequences (Fig. 7) and are very similar to those of the other transition metal P-type ATPases, particularly the various Cu²⁺ and Cd²⁺ ion pumps (5, 13, 14, 19, 36–39, 51, 52, 55–57) and the \textit{pRH439}-encoded \textit{H. pylori} P-type ATPase. The membrane spanning segments of the \textit{H. felis} pump are highlighted based on similarity to the \textit{CopA} P-type ATPase sequence of \textit{H. pylori}. As found for the \textit{H. pylori} pump, the P site is between H6 and H7 in the \textit{CopA} ATPase of \textit{H. felis}.

Putative TM sequences of the \textit{pRH948}-predicted \textit{H. pylori} \textit{CopA} pump were selected by various hydropathy-based algorithms and by their sequence homology to hydrophobic segments found in the \textit{H. felis} \textit{CopA} ATPase (Table 1 and Fig. 7). PCR-amplified copies of these segments of \textit{H. pylori} \textit{CopA} were assayed by coupled in vitro transcription-translation to determine their ability to membrane insert during translation. The N-terminal parts of the fusion proteins encoded by those vectors consist of either the first 101 (M0) or the first 139 (M1) amino acids of the \( \alpha \) subunit of the gastric proton pump followed by the putative TM domain of the \textit{CopA} ATPase. The C-terminal parts of the HK-M0–HK-M1 fusion vector with insert was translated in the presence of microsomal membranes. In turn, the presence of an insert encoding a stop transfer sequence was shown by inhibition of glycosylation of the HK-M1 fusion vector in the presence of microsomal membranes.

The first two hydrophobic regions, H1 (amino acid positions 84 to 104 [Fig. 8, lanes 1 to 4]) and H2 (amino acid positions 118 to 139 [Fig. 8, lanes 5 to 8]), were both signal anchor and stop transfer sequences. This should be the first pair of segments in the TM domain of this ATPase (Table 1). The signal anchor activity of H2 is weaker; this could be explained by a reverse orientation in the vector compared to its natural folding.

Sequences H3 (amino acid positions 159 to 180 [Fig. 9, lanes 1 to 4]) and H4 (amino acid positions 183 to 205 [Fig. 9, lanes 5 to 8]) are also both signal anchor and stop transfer sequences, indicating that H3 and H4 comprise the second pair of TM segments.
The next hydrophobic region following H4 in the *H. pylori* CopA ATPase is a sequence that has been named HX (amino acid positions 241 to 261 [Fig. 10, lanes 1 to 4]). Two segments of this region were translated in the vectors HK-M0 and HK-M1. Neither signal anchor nor stop transfer activity was found (Table 1). These results suggest that this region is not membrane inserted.

The putative TM region H5 (amino acid positions 340 to 363 [Fig. 10, lanes 5 to 8]) promoted a strong glycosylated band in the HK-M0 vector, similar to the one obtained with the HK-M1 vector control, and also performed as a stop transfer sequence in HK-M1. Six overlapping segments coding for the putative TM domain H6 (between amino acids 368 and 400 [Table 1]) were inserted in both HK-M0 and HK-M1. Most of them were able to promote partial glycosylation of the H/K ATPase β-subunit-derived sequences in the HK-M0 vector, as did the segment comprising amino acid positions 368 to 394 (Fig. 10, lanes 9 and 10). All of them prevented the glycosylation of the β-subunit sequences in the HK-M1 vector, therefore acting as a stop transfer sequence, which is the membrane insertion activity expected for this hydrophobic segment in the topological model (Fig. 10, lanes 11 and 12). These results suggest that H5 and H6 form the third pair of antiparallel helices of the CopA membrane domain.

The C-terminal region contains three main hydrophobic regions, HY, H7, and H8, which were expressed in the HK-M0 and HK-M1 vectors (Table 1). The results of the translation showed that the region HY (amino acid positions 629 to 646) could not act as a signal anchor sequence. When this region was translated in the HK-M1 vector, partial inhibition of glycosylation was observed. Data are shown in Fig. 11, lanes 1 to 4. The hydrophobic segment H7 (amino acid positions 679 to 703), which was expected to act as a signal anchor, was only a stop transfer sequence (Table 1; Fig. 11, lanes 5 to 8). However, when the H7 segment is extended upstream, therefore including the HY region (amino acid positions 629 to 703), translation in the HK-M0 vector showed a glycosylated product (Fig. 12, lane 2), indicating that the sequence preceding the
684-to-703 segment is important for the membrane insertion of H7 as a signal anchor sequence. The putative TM domain H8 (amino acid positions 708 to 728) acts as a stop transfer sequence, as expected from the eight-segment model of translation ion pumps (31) (Table 1; Fig. 11, lanes 9 to 12). The addition of the H8 coding region to the HY-H7 fragment suppressed the glycosylated HK-M0–CopA ATPase (amino acid positions 629 to 728) fusion product when expressed in the HK-M0 vector (Fig. 12, lane 4). Hence, the HY region helps to direct the membrane insertion of H7, and the hydrophobic H8 sequence returns the C terminus to the cytoplasmic side. Thus, H7 and H8 constitute the fourth TM segment pair of this ATPase.

Transmembrane segments of the CopA ATPase of H. felis and regions of high-level sequence homology. The corresponding TM segments of the H. felis ATPase were determined by hydrophobicity analysis and by their sequence similarity to the H. pylori ATPase. The data suggested that the H1 to H8 TM helices of the H. felis pump are contained between positions L89 and L108 (H1), F119 and G136 (H2), L156 and T177 (H3), G184 and G200 (H4), V337 and L357 (H5), A465 and M483 (H6), N666 and A685 (H7), and I690 and L710 (H8) (Fig. 2). The locations as well as the orientations of TM segments found in the H. felis pump were predicted to be identical to those of its H. pylori counterparts.

The overall identity of the amino acid sequences of the H. felis and H. pylori CopA ATPases was about 55%; considerable differences in degrees of identity were found when the corresponding TM segments were compared. The identities found were about 50% for H1 and H2, 65% for H3, 85% for H4, 70% for H5, 90% for H6 and H7, and about 75% for H8 (Fig. 2). Thus, the three N-terminal helices, H1, H2, and H3, have reduced identity while segments H4 to H8 exhibit greater amino acid identity, suggesting a role for the C-terminal TM segments in ion transport. There are other regions of high-level identity (>80%), namely in the N-terminal segment of ion binding around the GMXCCXXC motif, the small cytoplasmic loop between H2 and H3, some sequences following H4, the sequences around the conserved phosphorylation and ATP binding motifs, and sequences preceding the last putative pair of TM helices.

**DISCUSSION**

A DNA oligonucleotide mixture encoding the DKTGT(I/L)T phosphorylation consensus sequence had been used previously to isolate a putative transition metal P-type ATPase of *H. pylori* (31). Using a similar phosphorylation site screening strategy, we have isolated the copA operon from gene libraries of *H. pylori* 69A and a related gastric microorganism, *H. felis* (ATCC 49179). Cloning of the copA operons, therefore, provided evidence for the coexistence of at least two of the bacterial single-subunit P-type pumps in gastric *Helicobacter* species, the CopA ATPase and the pHH439-predicted pump (31). A third member of this class of membrane ATPases was observed in the genome of *H. pylori* 26695 (60).

The cop operons of both *H. pylori* and *H. felis* consist of only two genes, which encode a P-type pump, CopA, and a small peptide, CopP, with putative ion binding properties. Data obtained by insertion-deletion mutagenesis of the pHH439 P-type gene in *H. pylori* ATCC 49503 show that CopA may act as a copper export pump, as has been shown in *H. pylori* UA802 by using a sequence variant of the *H. pylori* *copA* gene (13) and in *Enterococcus hirae* for the CopB ATPase (38, 39, 56).

The predicted protein products of DNA sequences preceding the cloned *copA* gene are not homologous to proteins involved in ion transport or regulation of transport ATPase expression. In contrast, the *copP* gene immediately downstream of (in *H. pylori*) or overlapping with (in *H. felis*) *copA* DNA sequences predicts a protein homologous to the CopZ protein of the *Enterococcus hirae* Cu²⁺ ATPase operon (38). This suggests a role for the CopP peptide in regulation of *H. pylori* CopA ATPase expression rather than as a source of Cu²⁺ for the ATPase, as was suggested in a previous study (13). The presence of a CopZ-homologous protein in the *Helicobacter* copA operon might also suggest that there are similarities in regulation of cop gene expression in the gastric microorganisms and *Enterococcus hirae*. On the other hand, in *H. pylori* and *H. felis*, *copP* is located downstream of a unique CopA ATPase-encoding gene, and the cloned *copA* operons lack the equivalent of *copP* present in the enterococcal *copYZAB* operon, which is postulated to be a repressor of *cop* operon transcription (38). Given that the pHH439- and pHF8-predicted pumps analyzed here represent Cu²⁺ export ATPases, the *Helicobacter* operons also lack the physiological equivalent of the *Enterococcus hirae* copper import ATPase (38, 39, 55). However, there are additional genes contributing to transport of transition metal cations elsewhere in the genome of *H. pylori*, for example, the NixA Ni²⁺ transport protein (34, 60).

The ATPases predicted from the *copA* operon, 741 (*H. pylori* CopA) and 732 (*H. felis* CopA) amino acids in length, exhibit a strong overall sequence similarity to the previously studied 75-kDa membrane ATPase of *H. pylori* (31) and to the Cd²⁺ and Cu²⁺ P-type pumps of bacteria as well as mammalian cells (5, 36–39, 52, 62). As demonstrated for most of the members of this family of ion pumps, the cloned CopA ATPases have an N-terminal GMXCXXC ion binding motif (Cys box) and an intramembrane CPC sequence consistent with a role for this enzyme in transition metal transport (51, 52, 57). The GMXCXXC motif is a variant of a consensus sequence in the other putative transition metal ATPase isolated from *H. pylori* (31). The latter pump contains an N-terminal HXXHXXXCXXC ion binding motif with affinity for Ni²⁺ ions, indicating, as does the presence of clusters of cysteine and histidine residues in the ATP binding and phosphorylation loop of the latter enzyme (31), that the two CopA P-type ATPases expressed in *Helicobacter* species and the membrane
pump described previously have different ion specificities (31, 61).

Besides the data obtained by H. pylori copA gene knockout mutagenesis, evidence of a possible role for CopP in Cu²⁺-dependent gene regulation, and the homologies of the copA gene-predicted enzymes to the various Cd²⁺ and Cu²⁺ ATPases, there is additional preliminary evidence of a role for the cloned Helicobacter CopA pumps in binding as well as transport of copper ions. As shown in the data presented above, Cu²⁺, but not Co²⁺, Cd²⁺, or Mg²⁺, was significantly bound by the N-terminal peptide of the H. pylori 69A CopA ATPase predicted by vector pRH948. The selectivity of the peptide toward copper is most probably due to the presence of the GMXCGXC motif, as shown very recently for N-terminal domains of the human Wilson’s and Menkes Cu²⁺ transport ATPases (29). Weak binding of the H. pylori CopA peptide to Ni²⁺ and Zn²⁺ is probably not important for the physiology of H. pylori, since the CopA-deficient microorganism showed unchanged and high levels of resistance to both Ni²⁺ and Zn²⁺ (Table 2). The latter might be due to the existence of other proteins involved in metal ion resistance of H. pylori (31, 60). The N-terminal copper selectivity, as demonstrated for the H. pylori ATPase peptide, is in agreement with the increased sensitivity of H. pylori copA knockout mutants to Cu²⁺. The N-terminal region around the GMXCGXC box has a high degree of amino acid identity to various putative Cop ATPases, and a stretch of 22 amino acids in this region shows an identity of 91% when the CopA pumps of H. felis and H. pylori are compared, underlining a significant role of this domain in pump function. However, whether the N-terminal ion binding properties of P-type pumps contribute to the regulation of enzyme activity or to the transport of the ion itself is as yet unknown (28).

Structural features of these transition metal pumps must relate to their function as copper transport enzymes. Since the membrane domain contains the copper transport pathway, this domain was defined by in vitro translation scanning using membrane insertion detection vectors. In a previous study, this method was able, unequivocally, to detect eight membrane segments, H1 through H8, ordered pairwise along the polypeptide chain of the pRH439-predicted segments, H1 through H8, ordered pairwise along the polypeptide chain of the pRH439-predicted ATPase (31). The latter ATPase, with the highest degree of homology to Cd²⁺-transporting ATPases, contains three pairs of membrane-spanning helices (H1 to H6) in the N-terminal half of the enzyme and a fourth pair of antiparallel helices (H7 and H8) in the C-terminal region. To our knowledge, these results present the first experimental evidence that the membrane domain of Cu²⁺-transporting ATPases consists of eight TM segments. The additional hydrophobic peaks in the cloned H. pylori CopA ATPase, compared to the pRH439-encoded ATPase (31), did not act as signal anchors, and only the HY segment led to partial inhibition of glycosylation when translated in the HK-M1 vector. The H. felis CopA pump contains the corresponding H1 to H8 segments, as determined by hydropathy analysis and sequence similarity, but not the HX segment. The absence of this hydrophobic segment in the H. felis CopA ATPase is consistent with the finding that the HX segment of the corresponding H. pylori ATPase did not exhibit any membrane insertion properties. The HY segment was important for the membrane insertion properties of H7 in the H. pylori CopA ATPase and is retained in the H. felis CopA version. As in the other H. pylori P-type pump analyzed previously (31), H6 is followed by the large cytoplasmic energy transduction loop containing the phosphorylation site. On the basis of membrane topology, the cloned Helicobacter Cu²⁺ pumps, therefore, fall into a group of P-type ATPases containing eight TM segments.

The availability of CopA pumps from two distinct gastric Helicobacter species, H. pylori and H. felis, allowed a prediction of sequences related to the transport pathway for copper ions across the cytoplasmic membrane. Cysteine residues may play a role in ion transport, as found for the two cysteine residues located in the first TM region of the E. coli MerT mercuric ion transporter (35). In CopA, cysteines are thought to participate in Cu²⁺ binding sites and therefore may be involved in the transport of the ion through the membrane. A feature of these Helicobacter Cu²⁺ pumps is that they contain seven (for H. felis) or eight (for H. pylori) cysteine residues. Five of these cysteines are located at conserved positions in TM helices. These cysteines are in H4, in H6 as part of the conserved CPC motif, and in H7. When the Enterococcus hirae CopAB pumps (37) and the cloned Cu²⁺ pumps of H. pylori and H. felis are compared, only the cysteine residue in the CPX motif of H6 is conserved among the transition metal pumps, bringing into question the role of the other conserved cysteine residues present in the membrane domain of both the H. pylori and H. felis CopA pumps. However, the TM segments H7 and H8, as well as H4, H5, H6, and in part H3, are well conserved between the two ATPases. Along with the placement of the homologous cysteines, this conservation could be taken as preliminary evidence that TM segments H4 to H8 constitute the core structure of the ion transport domains of these ATPases. The first two TM sequences show less homology to each other and to the other P-type ATPases of H. pylori (31, 60). Therefore, H1 and H2 of the cloned CopA pumps, which represent the two additional N-terminal TM segments found in all transition metal ATPases, and perhaps H3 may be situated peripherally to this core structure, interacting mainly with phospholipid, which would account for their relative lack of conservation.

In conclusion, H. pylori and H. felis employ conserved mechanisms of copper resistance. These mechanisms include expression of a bicistronic copAP operon encoding a transition metal pump containing eight TM helices, CopA, and the peptide CopP, the latter most probably being a Cu²⁺-binding protein involved in copAP operon expression. The CopA ATPase, which provides the structural and functional basis for P-type Cu²⁺ export in Helicobacter species, uses conserved motifs such as the N-terminal domain with Cu²⁺-binding properties and conserved regions in the energy transduction domain. The CopA membrane domain shows the highest level of sequence identity in H4, H5, H6, and H7-H8 as well as in stretches of H3, suggesting that these six segments of the CopA ATPase may provide the TM pathway for copper ions across the H. pylori and H. felis inner membranes.

ACKNOWLEDGMENTS

We thank Anita Buhmann, Marion Eisenhauer, and Marina Rentsch for excellent technical assistance.

This work was supported in part by the Bundesministerium für Forschung und Technologie (BMTT grant 514 4003 031077) and in part by U.S. Veterans Administration research funds (SMI) and National Institutes of Health grants DK40615, DK41301, and DK17294.