JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 674–683
0021-9193/93/030674-10$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 175, No. 3

# Cloning, Nucleotide Sequence, and Expression of a Gene Encoding an Adhesin Subunit Protein of *Helicobacter pylori*

DOLORES G. EVANS,[1]* TUOMO K. KARJALAINEN,[2] DOYLE J. EVANS, JR.,[1] DAVID Y. GRAHAM,[1] AND CHAO-HUNG LEE[2]

*Department of Medicine, Baylor College of Medicine and Veterans Affairs Medical Center, Houston, Texas 77030,[1] and Department of Pathology, Indiana University School of Medicine, Indianapolis, Indiana 46202[2]*

Received 27 August 1992/Accepted 23 November 1992

**Gene *hpaA*, which codes for the receptor-binding subunit of the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin (NLBH) of *Helicobacter pylori*, was cloned and sequenced. The protein expressed by *hpaA*, designated HpaA, was identified as the adhesin subunit on the basis of its fetuin-binding activity and its reactivity with a polyclonal, monospecific rabbit serum prepared against NLBH purified from *H. pylori*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and Western blots (immunoblots) showed that the cloned adhesin has the same molecular weight (20,000) as that found on *H. pylori*. Also, HpaA contains a short sequence of amino acids (KRTIQK) which are all either identical or functionally similar to those which compose the sialic acid-binding motif of *Escherichia coli* SfaS, K99, and CFA/I. Affinity-purified antibody specific for a 12-residue synthetic peptide that included this sequence blocked the hemagglutinating activity of *H. pylori* and was shown by immuno-gold electron microscopy to react with almost transparent material on unstained *H. pylori* cells, which is consistent with previous observations concerning the location and morphology of the NLBH.**

Numerous studies have established that *Helicobacter pylori* is the etiologic agent of type B gastritis (5, 17, 33, 34). Mounting epidemiological evidence implicates chronic *H. pylori* gastritis in the development of gastric and duodenal ulcer disease (1, 22, 32, 36, 50) and also gastric carcinoma (10, 29). *H. pylori* infection has a worldwide distribution; it is more prevalent in developing countries and in economically disadvantaged people in more developed countries (5, 17, 39). Although little is known about the mechanism(s) by which *H. pylori* infection leads to gastroduodenal disease, it has been shown that this microaerophilic organism preferentially adheres to and colonizes mucus-secreting gastric epithelial cells (1, 5, 20, 32, 36). Like other bacteria which colonize mucosal epithelia, *H. pylori* adheres to sialic acid-rich macromolecules that are exposed on the mucosal cell membrane (6). We identified a fibrillar *N*-acetylneuraminyllactose (NL)-binding hemagglutinin (NLBH) on *H. pylori* as a putative colonization factor (14) and obtained serological evidence for its production in vivo (15); we also identified the role of this molecule in the adherence of *H. pylori* to mammalian cells in tissue culture (13). The NLBH could easily account for the finding of Slomiany et al. (47) that GM3 ganglioside inhibits binding by *H. pylori*. Slomiany et al. (47) also showed that binding was inhibited by lactosylceramide sulfate, which presumably is the novel glycerolipid receptor described by Lingwood et al. (28). Recently, Saitoh et al. (42) identified both an NL-containing moiety (GM3-ceramide) and sulfated lactosylceramide as *H. pylori* receptors that are present in human gastric mucosa; their results indicated the existence of two different *H. pylori* ligands corresponding to these two receptors.

Our interest is in defining the molecular mechanisms by which *H. pylori* colonization of the gastric epithelium leads to serious gastroduodenal disease. Here we report cloning and sequencing of the *H. pylori* adhesin gene *hpaA* and characterization of the *hpaA* product, adhesin subunit protein HpaA, which is responsible for the binding specificity of the NLBH.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Escherichia coli* K-12 strain DH5α (3) was used as the host for cloning; strain HB101 (8) was used for expression of cloned DNA; strain XL1-Blue (8) was used for lambda Zap II and for in vivo excision of pBluescript phagemid; and *E. coli* JM109 (51) was used for preparation of single-stranded phage for sequencing. For plasmid isolation and recombinant DNA experiments, *E. coli* strains were grown in Luria-Bertani medium (43) at 37°C with vigorous aeration. For infection with lambda Zap II, strain XL1-Blue cells were grown in either Luria-Bertani or NZY medium supplemented with 10 mM $MgSO_4$ and 0.2% maltose; strain JM109 was grown in 2× YT medium (43). Cultures were incubated at 30°C with vigorous shaking. *H. pylori* was grown as described previously (18). Antibiotics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 100 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 12.5 μg/ml.

**Isolation of genomic DNA.** *H. pylori* DNA was isolated as described by Majewski and Goodwin (31).

**Purification of the *H. pylori* 20K adhesin and preparation of anti-adhesin antibody.** *H. pylori* cells from 20 blood agar plates (13) were extracted with 20 ml of 1.0% *N*-octylglucose in 0.05 M Tris-Cl (pH 8.0). The extract, which had both urease and fetuin-binding activities, was dialyzed against phosphate-buffered saline (PBS). The precipitate thus obtained was dissolved in 5.0 ml of a solution containing 1.0% *N*-octylglucose and 5.0% β-mercaptoethanol in 0.05 M Tris-Cl buffer and then boiled for 10 min. The resulting small precipitate was discarded, and the protein in the supernatant was purified by fast-pressure liquid chromatography (Phar-

* Corresponding author.

macia-LKB, Piscataway, N.Y.), using a Sepharose 6 column. Fractions eluted with the Tris buffer described above containing 1.0% *N*-octylglucose but no β-mercaptoethanol were assayed for both urease and fetuin-binding activities. Only one peak fraction, which had a low optical density at 280 nm ($OD_{280}$), had high fetuin-binding activity; a dilution of 1:800 of the peak fraction having an $OD_{280}$ of 0.050 typically gave 1.0 enzyme-linked immunosorbent assay (ELISA) optical density unit. This peak fraction had no urease activity even when it was assayed undiluted. An analysis of the peak fraction by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) revealed a single protein with an estimated molecular weight of 20,000 (20K) after silver staining.

Polyclonal specific antibody against this protein was produced by hyperimmunization of rabbits with protein purified in this manner. C. Sciortino kindly provided a monoclonal antibody prepared by injecting mice with *H. pylori*.

**Production of antibody specific for a sequence of amino acids including the receptor-binding motif of HpaA.** The peptide Cys-Leu-Arg-Pro-Asp-Pro-Lys-Arg-Thr-Ile-Gln-Lys-Lys was synthesized (Multiple Peptide Systems, San Diego, Calif.) and conjugated to a protein carrier (keyhole limpet hemocyanin) via an N-terminal cysteine residue added for this purpose. Rabbits were hyperimmunized with the conjugate by using initial and three booster injections. A high-titer serum was obtained, as demonstrated by an ELISA in which the purified peptide was used as the antigen. Specific anti-peptide antibody was purified from the immune serum by affinity chromatography, using the synthetic peptide covalently attached to a ProtOn column (kit no. 1; Multiple Peptide Systems).

**Immunolabeling and electron microscopy of *H. pylori* cells.** *H. pylori* 8826 cells were grown as previously described (14). A cell suspension (15 μl) was placed on carbon-stabilized nitrocellulose film copper specimen grids, and the excess fluid was removed after 3 min. Reagents (15 μl each) were added in the following order: 2% bovine serum albumin in PBS; affinity-purified anti-peptide antibody (diluted 1:200 in PBS) or, as control, preimmune rabbit serum; 2% bovine serum albumin in PBS; goat anti-rabbit immunoglobulin G labeled with 10-nm-diameter gold particles (Sigma Chemical Co.); and finally glass-distilled water. Grids were observed with a Philips CM10 PW6020 transmission electron microscope.

**Construction of *H. pylori* genomic library.** An *H. pylori* expression library was constructed in lambda Zap II vector obtained from Stratagene, La Jolla, Calif., by using standard procedures (43). Genomic DNA that was partially digested with *Eco*RI was fractionated on a sucrose density gradient, and 5- to 10-kb fragments were isolated. A 0.2-μg portion of the insertion DNA was ligated with 1.0 μg of *Eco*RI-digested lambda arms. Following in vitro packaging, the library was titrated by infecting strain XL1-Blue cells with aliquots of packaged phage and was plated onto indicator plates containing isopropyl-β-D-thiogalactopyranoside (IPTG) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The ratio of nonrecombinant phage to recombinant phage was determined to be 1:500. The titer of the recombinant library was calculated to be $2.5 \times 10^7$ PFU/ml.

**Screening of *H. pylori* genomic library with antibodies.** The amplified library, which had a titer of $4 \times 10^9$ PFU/ml, was replated onto 20 90-mm plates (50,000 recombinant bacteriophage plaques per plate) and was lifted onto nitrocellulose filters. The filters were screened with a 1:3,500 dilution of monospecific anti-adhesin serum. The antigen-antibody complexes were visualized with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma Biochemical Co., St. Louis, Mo.) by using as the enzyme substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Once a positive phage clone was identified, an agar plug containing the plaque was picked, and the phage were eluted. The process of infecting, replating, and screening with antibodies was repeated until plaque purity was obtained.

To eliminate nonspecific reactivity, the serum used for screening was adsorbed first with heat-killed (100°C for 30 min) whole strain XL1-Blue cells and whole-cell lysate obtained by sonication and then with a lysate consisting of strain XL1-Blue cells infected with lambda Zap II. Antigen-antibody complexes, including phage, were removed by centrifugation at 26,000 × *g* for 2 h. The serum was then sterilized by filtration. The specificity of the antibody was confirmed by a dot immunoassay, using as the antigen dilutions of whole cells or whole-cell lysates of *H. pylori* (positive control) or strain XL1-Blue cells that were both not infected and infected with lambda Zap II (negative controls). The adsorbed serum was not reactive with the negative controls at all of the dilutions tested but gave strong reactions at all antigen dilutions with the positive control, even at a 1:3,500 dilution.

**In vivo excision of phagemid pBluescript from lambda Zap II.** In vivo excision of pBluescript containing insert DNA from lambda Zap II was accomplished by infecting strain XL1-Blue simultaneously with lambda Zap II containing insertion DNA and helper phage R408, as described by Short et al. (46).

**DNA preparation, manipulation, and analysis.** Plasmid DNA was isolated by the alkaline lysis method (4) and was purified by centrifugation in a cesium chloride-ethidium bromide gradient (43). Restriction enzyme digestions and ligations with T7 ligase were performed as recommended by the enzyme manufacturer (Bethesda Research Laboratories). Restriction mapping of the insertion in pHPA was performed with *Hin*dIII, *Cla*I, *Xba*I, *Xho*I, *Sma*I, *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *Bgl*II, *Sac*I, and *Kpn*I. Restriction fragments were electrophoresed in 1.0% agarose gels and were stained with ethidium bromide. Transformations were performed by using the polyethylene glycol method described by Chung et al. (9).

**Plasmid constructs.** pHPA is a pBluescript SK(+) phagemid with a 2.8-kb *Hin*dIII-*Eco*RI fragment of *H. pylori* genomic DNA containing the genetic determinant for the 20K adhesin (Fig. 1). This phagemid was derived from an antibody-positive lambda Zap II clone by in vivo excision as described above. Subclones of pHPA, designated pHPA22 and pHPA23, were constructed as follows. pHPA was digested with *Sma*I, resulting in excision deletion of a 1.8-kb *Sma*I fragment. The 1.8-kb *Sma*I fragment was eluted from the agarose and cloned into pBluescript SK(+), resulting in subclone pHPA23. The remaining vector and the 1.0-kb insertion DNA were religated, resulting in subclone pHPA22. The same approach was used to clone the 1.0-kb *Sac*I-*Kpn*I fragment of pHPA (pHPA24), the 0.4-kb *Sma*I-*Kpn*I fragment (pHPA25), the 1.4-kb *Hin*dIII-*Kpn*I fragment (pHPA26), and the 1.4-kb *Kpn*I-*Sma*I fragment (pHPA27).

**DNA sequencing.** Several overlapping DNA fragments of the adhesin region cloned into either pBluescript or pTZ phagemid (U.S. Biochemical Corp., Cleveland, Ohio) vectors were sequenced. Single-stranded DNA phagemid templates were prepared by the polyethylene glycol method (44). The sequence was determined by the dideoxynucle-

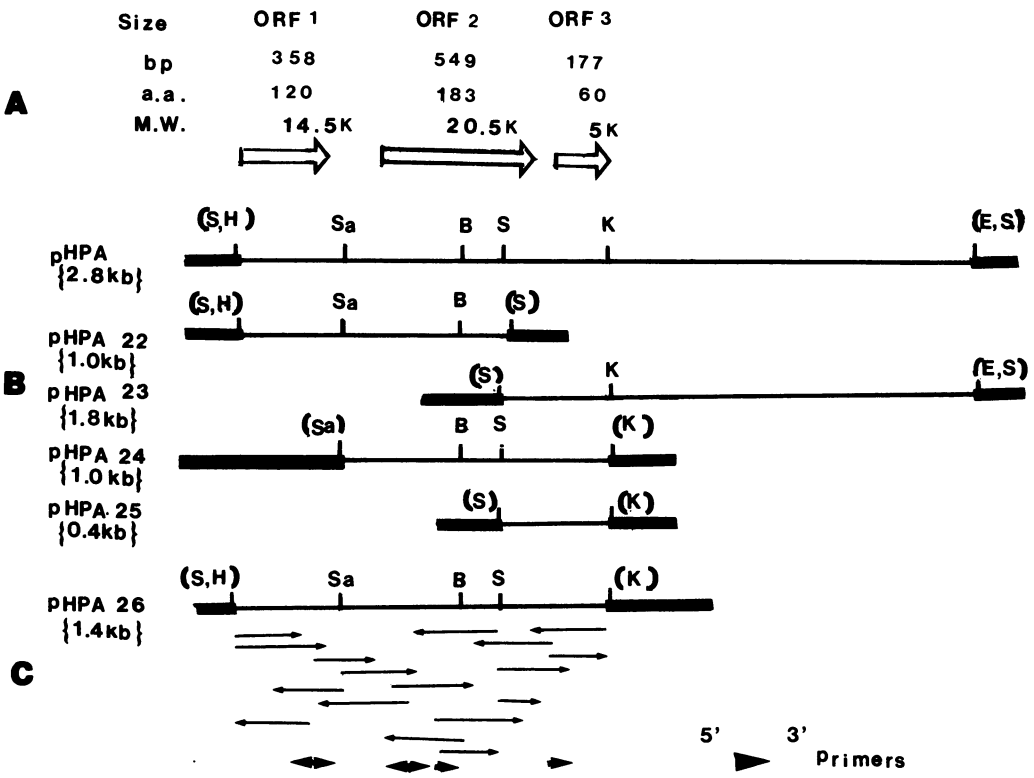


FIG. 1. Linear restriction maps of pHPA and its subclones, constructed as described in the text. (A) Schematic representation of three ORFs deduced from the nucleotide sequence analysis, including their orientations, their sizes (in nucleotides and amino acids [a.a.]), and their calculated molecular weights (M.W.). (B) Restriction maps of pHPA and subclones pHPA22, pHPA23, and pHPA26 in pBluescript SK and pHPA24 and pHPA25 in pBluescript KS. (C) Strategy for DNA sequencing, as described in the text. The values in braces are the sizes of the *H. pylori* DNA fragments inserted into the vector pBluescript (thick lines). Restriction sites: E, *Eco*RI; S, *Sma*I; H, *Hin*dIII; Sa, *Sac*I; B, *Bgl*I; K, *Kpn*I. Parentheses indicate that the restriction sites originated from the vector.

otide chain termination method (44), using a Sequenase kit (U.S. Biochemical Corp.). In addition, portions of the adhesin region were sequenced by using synthetic oligonucleotide primers and double-stranded DNA as the template (52).

**SDS-PAGE and Western blot analysis of proteins.** The total proteins expressed by *H. pylori* or cloned *H. pylori* DNA in *E. coli* were analyzed by standard SDS-PAGE and Western blot (immunoblot) techniques (43). A 5-ml portion of *E. coli* HB101 cells containing recombinant plasmids was grown in 2× YT medium at 37°C for 2 h, and then 1.0 ml of the culture was withdrawn, collected by centrifugation, and resuspended in SDS-PAGE loading buffer. IPTG was added to the remaining 4.0 ml of culture to a final concentration of 1.0 mM; 1.0-ml samples were processed as described above after 4 h. Proteins were resolved by electrophoresis in a SDS–12% polyacrylamide gel (27) and were stained with Coomassie blue. A gel run in parallel was electrotransferred onto nitrocellulose filters. The detection of adhesin-antibody complexes was performed as described under "Screening of *H. pylori* genomic library with antibodies."

**Expression of cloned *H. pylori* proteins by using T7 RNA polymerase.** T7 RNA polymerase-induced transcription and subsequent translation of the adhesin gene were accomplished by using the in vivo expression system of Tabor and Richardson (49). pBluescript or pTZ19R carrying *H. pylori* sequences downstream from the T7 promoter were transformed into *E. coli* K38 carrying pGP-1 encoding T7 RNA polymerase under the control of the lambda PL promoter. After overnight incubation at 30°C, the cultures containing the plasmids were diluted to an $OD_{590}$ of 0.4. A 1-ml portion of bacteria was then pelleted, washed in M9 medium, and resuspended in 5.0 ml of M9 medium supplemented with a 19-amino-acid mixture (lacking methionine) at a concentration of 0.02%. After 1 h at 30°C, induction of transcription was initiated by shifting the temperature to 42°C. *E. coli* RNA polymerase was inhibited after 20 min by adding rifampin (200 μg/ml for 10 min). After an additional 20 min at 30°C, 0.5 ml of cells was pulsed for 5 min with 10 μCi of $^{35}$S-labeled methionine at 30°C. The cells were then collected and resuspended in 150 μl of SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE, using 15% polyacrylamide gels. The gels were fixed and examined by fluorography, using En$^3$Hance (New England Nuclear Corp., Boston, Mass.).

**Fetuin ELISA.** Receptor-binding activity was determined by the fetuin-ELISA method, essentially as described previously (14). Microtiter wells were coated with 1.0 μg of fetuin per ml in 0.05 M Tris-Cl (pH 8.0) for 18 h at 37°C. The wells were blocked with 1.0% bovine serum albumin in PBS before the addition of test samples. Antigen-antibody complexes were detected by an ELISA in which anti-adhesin serum was used.

Whole-cell lysates (1.0 mg of protein) of IPTG-induced *E. coli* HB101 carrying the recombinant plasmids or pBluescript without an insertion were assayed. The positive controls consisted of an *H. pylori* 8826 whole-cell lysate and 1.0 μg of purified 20K adhesin. Results were expressed as the

$OD_{450}$ obtained 1 h after the addition of alkaline phosphatase substrate.

**Southern hybridization and labeling of the DNA probe.** Standard procedures were used to transfer restriction enzyme-digested DNA to nitrocellulose membranes (43). Prehybridization and hybridization of baked filters were done at 68°C for 12 h in a solution containing 5× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate [43]), 0.5% blocking reagent, 0.1% *N*-lauroyl sarcosine, and 0.2% SDS. The probe was purified and labeled by the random labeling method, using [$^{32}$P]dCTP and a kit purchased from U.S. Biochemical Corp. or dioxygenin-dUTP (Genius kit; Boehringer Mannheim Biochemicals). Following high-stringency washing (two washes with 6× SSC containing 0.5% SDS [each for 15 min at 25°C], followed by two washes with 1× SSC containing 0.5% SDS [each for 30 min at 37°C] and then two washes with 0.1× SSC containing 1.0% SDS [each for 30 min at 55°C]), the hybridization results were visualized by color development or autoradiography.

**Nucleotide sequence accession number.** The nucleotide sequence of the *H. pylori* adhesin gene region has been deposited in the EMBL/GenBank Library under accession number X61574.

## RESULTS

**Cloning of the *H. pylori* 20K adhesin gene.** Previously, we identified and partially purified a fibrillar adhesin from *H. pylori* which binds NL-containing receptors on erythrocytes and epithelial cells in tissue culture (13, 14). Preliminary experiments showed that the binding activity of the adhesin is associated with a 20K protein and that serum from hyperimmunized rabbits detected only a 20K protein in immunoblots of *H. pylori* whole-cell lysate proteins resolved by SDS-PAGE. This hyperimmune serum also blocked hemagglutination of erythrocytes by intact *H. pylori* cells.

To further characterize the *H. pylori* 20K protein, we sought to isolate the genetic determinant for this protein from the *H. pylori* chromosome; to do this, an *H. pylori* genomic expression library was constructed in lambda Zap. Approximately $10^6$ plaques were screened by using anti-adhesin serum. A total of 50 positive plaques were obtained. Ten positive plaques were randomly picked and plaque purified. Since one of the *Eco*RI sites was destroyed during cloning, the presence of DNA fragments was confirmed by *Eco*RI-*Hin*dIII digestion of plasmid DNA. Analysis of the DNA obtained after in vivo excision of pBluescript phagemid from each of the 10 plaques revealed that each of the positive clones contained an insert, ranging in size from 2.8 to 4.5 kb. Southern hybridization analysis in which a 159-bp *Sma*I-*Bgl*II fragment derived from the 2.8-kb *Eco*RI-*Hin*dIII *H. pylori* DNA insertion was used as the probe showed that all 10 randomly picked positive clones contained the same insert. The probe also hybridized with chromosomal DNAs isolated from other *H. pylori* strains but did not hybridize with an antibody-negative clone or with the pBluescript vector.

The plasmid containing the 2.8-kb insert was named pHPA (*Helicobacter pylori* adhesin) and was selected for further study.

**Restriction map of pHPA and its subclones.** Restriction mapping of the insert in pHPA was performed. This insert contains only one *Sma*I site, which is 1.0 kb from one end of the insertion, one *Bgl*II site, one *Sac*I site, and one *Kpn*I site (Fig. 1); the other enzymes tested did not cleave within the insert. To more precisely define the region of the insert that encodes the adhesin, several subclones of pHPA were constructed (Fig. 1). pHPA23 was constructed by deleting a 1.8-kb *Sma*I fragment from pHPA. The remaining vector and the 1.0-kb insert DNA up to the *Sma*I site were religated and constitute pHPA22. pHPA24 contains a 1.0-kb *Sac*I-*Kpn*I fragment from pHPA; pHPA25 contains a 0.4-kb *Sma*I-*Kpn*I fragment; pHPA26 contains a 1.4-kb *Hin*dIII-*Kpn*I fragment; and pHPA27 contains a 1.4-kb *Kpn*I-*Sma*I fragment in the pBluescript vector.

**Detection of proteins expressed by pHPA and its subclones.** If the insert in pHPA contains the gene that codes for the 20K adhesin, the recombinant plasmid should direct the expression of a protein of that size that is detectable by the anti-adhesin antibody. The proteins expressed by pHPA and its subclones present in whole-cell lysates of *E. coli* HB101 were analyzed by SDS-PAGE (Fig. 2A) and Western blotting (Fig. 2B). It is evident from the immunoblots (Fig. 2B) that pHPA (lane 2) and pHPA22 (lane 4) but not pHPA23 (lane 3) expressed a protein with an estimated molecular weight of 20,000. The same protein was detected by the monospecific anti-adhesin antibody in *H. pylori* whole-cell proteins (lane 5) and in the control preparation consisting of purified 20K adhesin (lane 6). The specificity of the anti-adhesin antibody was confirmed by the fact that none of the *E. coli* whole-cell lysate proteins containing pBluescript without the insert and no other *H. pylori* lysate protein or other protein purified from *H. pylori* reacted with the antibody (lanes 7 and 8). Thus, the genetic determinant for the 20K adhesin of *H. pylori* is apparently located on the 1.0-kb *Hin*dIII-*Sma*I fragment of pHPA22.

**Binding of the 20K protein expressed by pHPA and its derivatives to fetuin.** We assayed the binding of the protein expressed by pHPA and subclones pHPA22 and pHPA23 to fetuin immobilized on microtiter plate wells by the fetuin-ELISA technique (14). The specificity of binding to NL was confirmed, since none of the samples bound to microtiter wells coated with asialo-fetuin. The results are presented in Table 1.

Whole-cell lysates of *E. coli* HB101 carrying pHPA or pHPA22 bound to fetuin, as did the purified 20K adhesin and whole-cell protein of *H. pylori* 8826 (the positive control). Since the whole-cell proteins of *E. coli* HB101 carrying the vector without an insert or carrying pHPA23 did not bind to fetuin, it could be concluded that the protein expressed by pHPA and pHPA22 includes the fetuin-binding adhesin.

To test the antigenic similarity between proteins expressed by cloned *H. pylori* DNA and purified 20K *H. pylori* adhesin, a blocking ELISA was performed. Rabbit anti-adhesin serum was incubated with an equal volume of a test sample (1.0 mg of protein) before the preparation was added to wells containing the purified adhesin bound to fetuin-coated plates. The controls consisted of preparations containing serum plus diluent buffer or serum plus the purified 20K adhesin. Table 2 shows the results. The proteins expressed by pHPA and by pHPA22 efficiently blocked the reaction of anti-adhesin with the purified 20K antigen. The protein expressed by pHPA23 blocked the antigen-antibody reaction poorly, whereas the proteins expressed by pBluescript without an insert did not block the reaction.

**Nucleotide sequence of the adhesin region.** The nucleotide sequences of both strands and of all of the overlapping fragments containing the region encoding the adhesin were determined for the 1.4-kb *Hin*dIII-*Kpn*I fragment of pHPA26 (Fig. 1). The nucleotide sequence is shown in Fig. 3. The 1,400 bp spanning the adhesin region was analyzed to determine the presence of open reading frames (ORFs);

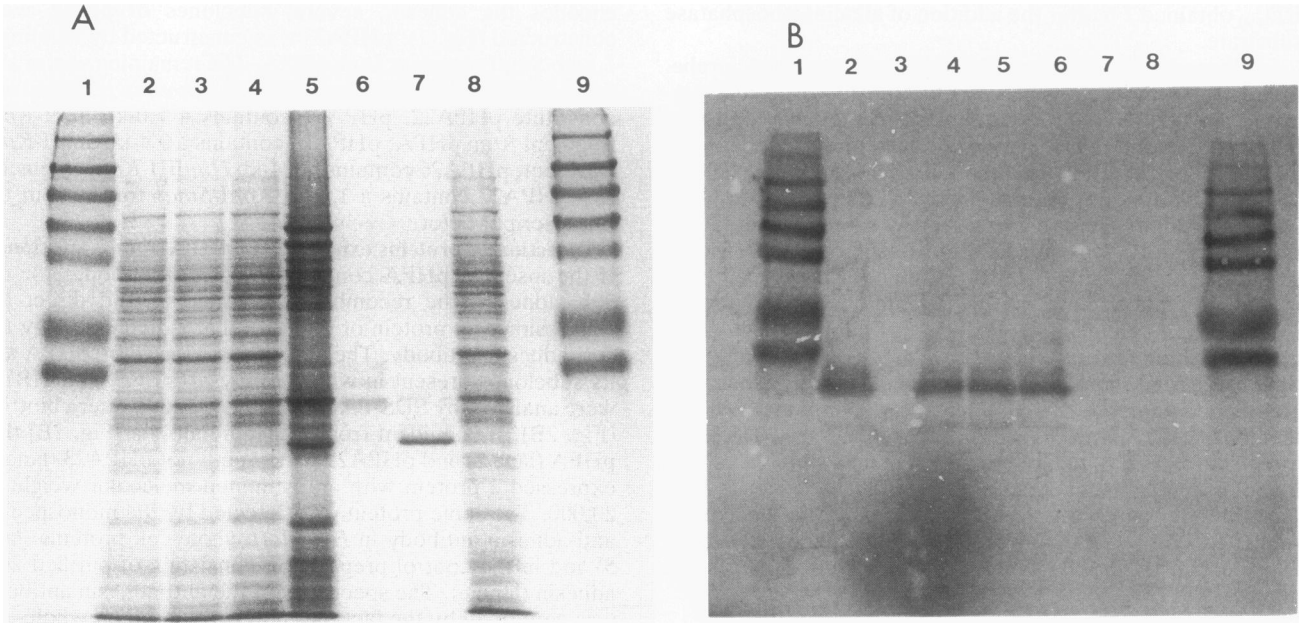


FIG. 2. (A) SDS-PAGE (12% acrylamide) gel of total proteins (20 μg) from recombinant clones in *E. coli* HB101 stained with Coomassie blue. (B) Corresponding immunoblot developed with a 1:2,000 dilution of monospecific anti-*H. pylori* adhesin serum. Lanes 2, pHPA; lanes 3, pHPA23; lanes 4, pHPA22; lanes 5, *H. pylori* total protein (30 μg); lanes 6, partially purified *H. pylori* adhesin (1 μg); lanes 7, another purified *H. pylori* protein (2 μg); lanes 8, vector pBluescript in *E. coli* HB101. Lanes 1 and 9 contained molecular weight standards having molecular weights of 180,000, 116,000, 84,000, 58,000, 49,000, 36,500, and 26,500.

three ORFs encoded by the same strand were found (Fig. 1). No ORFs of any significant length were found on the reverse strand complementary to the sequence of the strand shown in Fig. 3. These ORFs were designated ORF1, ORF2, and ORF3. Two ORFs, ORF2 and ORF3, begin with a characteristic ATG start codon. ORF1, which contains 357 bp starting at position 1, has coding potential for a 14.5K protein (Fig. 3). Analysis of the ORF1 sequence reveals several methionine codons; however, none was preceded by a Shine-Dalgarno ribosomal binding site. From this finding we predicted that ORF1 is an incomplete sequence of a larger protein which is truncated at the *Eco*RI site in the 2.8-kb cloned DNA fragment.

The ORF2 sequence starts at position 576, contains 549 bp, and has coding potential for a 20.5K protein. ORF2 is the only ORF preceded by a site similar to an *E. coli* consensus ribosomal binding (Shine-Dalgarno) sequence, AGGA (45). ORF2 is also preceded by two sequences resembling the TTGACA (−35) and TATAAT (−10) *E. coli* promoter consensus sequences (41), as shown in Fig. 3. Also, the transcriptional termination codon of ORF2 is followed by an 8-bp inverted repeat 12 bases downstream. This motif might constitute a weak stem-loop structure typical of a rho-dependent transcriptional termination signal.

ORF3 starts at position 1177 (Fig. 3) and has coding potential for a 5K polypeptide. The initiation codon is not preceded by a ribosomal binding site or a putative promoter consensus sequence. The product of ORF3 is not likely to be the adhesin since its entire sequence lies within pHPA23, which expresses proteins that are not reactive with anti-adhesin antibody (Fig. 2) and do not bind to fetuin (Table 1).

The mature HpaA protein has several hydrophobic regions. The major hydrophobic regions are from amino acid residue 18 to amino acid residue 36, from amino acid residue 120 to amino acid residue 130, and at the carboxyl terminus (amino acid residues 159 to 174).

TABLE 1. Fetuin binding of proteins expressed by *E. coli* HB101 carrying pHPA and its subclones[a]

| Prepn | $OD_{450}$ (mean ± SD) |
|---|---|
| pHPA | 0.559 ± 0.044 |
| pHPA23 | 0.053 ± 0.015 |
| pHPA22 | 0.582 ± 0.054 |
| Whole-cell lysate of *H. pylori* | 0.728 ± 0.021 |
| pBluescript (no insertion) | 0.043 ± 0.013 |
| Purified adhesin | 1.000 ± 0.025 |

[a] Plasmid proteins in *E. coli* HB101 were induced with 1.0 mM IPTG. Whole-cell lysates were assayed by using 1.0 mg of protein; 20K adhesin was assayed by using 0.1 μg of protein. Anti-adhesin rabbit antibody was used at a final dilution of 1:2,000. All of the samples were tested on asialo-fetuin-coated ELISA plates and were negative for binding ($OD_{450}$, <0.05).

TABLE 2. Antigenic similarity of the proteins expressed by pHPA, pHPA22, pHPA23, and pBluescript to purified 20-kDa *H. pylori* adhesin, as determined by a blocking ELISA[a]

| Addition to anti-adhesin antibody (1:2,000) | $OD_{450}$ (mean ± SD) |
|---|---|
| None | 0.61 ± 0.08 |
| pHPA | 0.05 ± 0.01 |
| pHPA22 | 0.04 ± 0.02 |
| pHPA23 | 0.22 ± 0.02 |
| pBluescript | 0.58 ± 0.06 |
| Purified 20-kDa adhesin | 0.05 ± 0.02 |

[a] Fetuin-coated microtiter plate wells were first exposed to purified 20-kDa adhesin at a concentration of 0.01 μg of protein per ml. Whole-cell lysates of *E. coli* HB101 containing different plasmids were prepared 4 h after IPTG induction. Samples were adjusted to the same protein concentration.

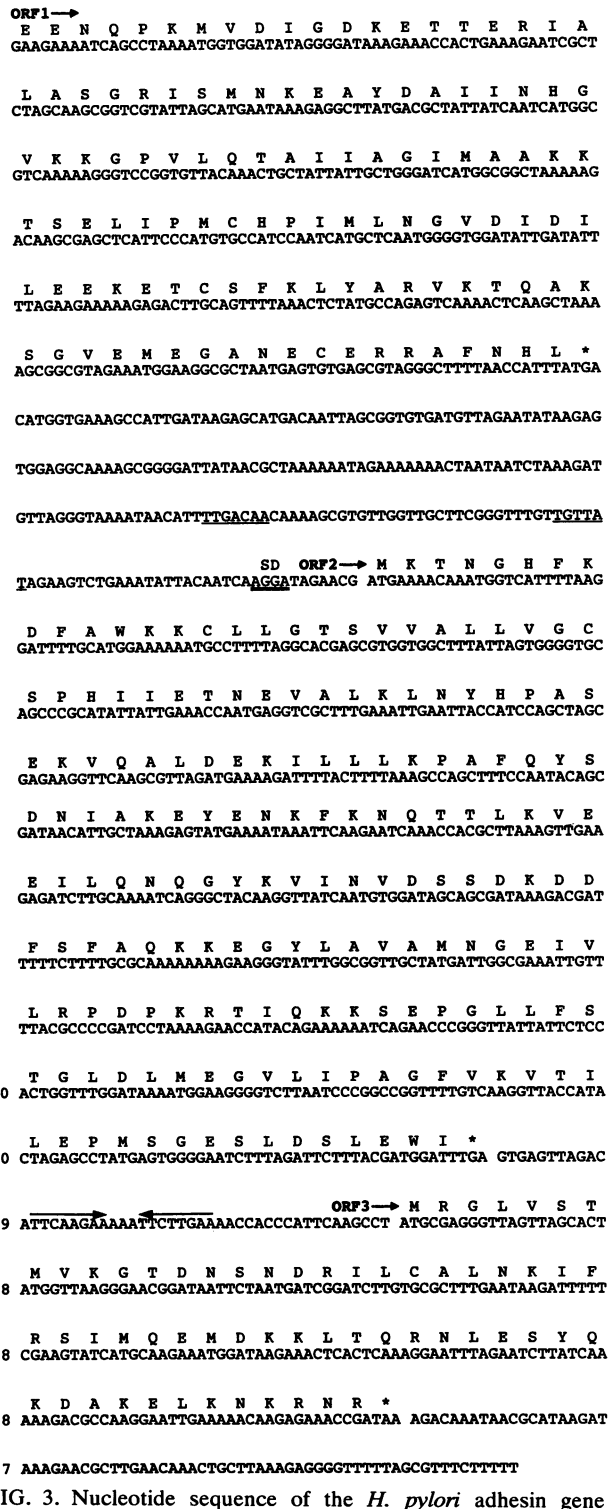


FIG. 3. Nucleotide sequence of the *H. pylori* adhesin gene region. The numbers indicate the nucleotide and amino acid positions. The deduced amino acid sequences for ORF1 (positions 1 to 357), ORF2 (positions 576 to 1124), and ORF3 (positions 1177 to 1353) are shown below the base sequence. The putative Shine-Dalgarno ribosomal binding sequence (SD) is underlined with two lines; the promoterlike sequences are underlined with one line. The arrows above the sequence indicate the position of a possible stem-loop structure resembling a rho-dependent transcriptional stop signal.

**Analysis of peptides expressed by pHPA and its subclones with the T7 RNA polymerase.** To determine which ORF codes for the adhesin, pHPA and subclones pHPA22 and pHPA24, which contained overlapping DNA fragments (Fig. 1), were expressed by using the in vivo T7 RNA expression system. It is evident from the fluorography that the ORF1 sequences present in pHPA and pHPA22 expressed a peptide with an apparent molecular weight of 15,000 (Fig. 4A, lanes 3 and 4). A peptide with the same molecular weight was expressed by the same DNA fragments of pHPA and pHPA22 cloned in a different vector, pTZ18u (Fig. 4A, lanes 6 and 7). This 15K peptide was not expressed by pHPA24, which carries a DNA fragment in which the ORF1 sequence has been deleted (Fig. 4A, lanes 1 and 5), indicating that the 15K peptide is the product of ORF1.

pHPA and pHPA22, which contain ORF1 and ORF2 (Fig. 1), expressed both a 15K peptide and a 20K peptide (Fig. 4A, lanes 3 and 4), whereas pHPA24, which contains only ORF2, expressed only the 20K peptide. Immunoblot analysis showed that only the 20K peptide was recognized by the anti-adhesin serum (Fig. 4B). This indicates that ORF2 codes for the adhesin. The gene encoding the 20K protein was named *hpaA*, and its product was named HpaA. It should be noted that extra bands, including one near the 20K band (Fig. 4A, lanes 1, 3, and 5 through 7), most likely represent false initiation or termination products since transcription-translation in the presence of 9% ethanol, which inhibits peptide processing (12, 37), did not change the molecular weights of the products expressed (data not shown).

**Binding of pHPA24-expressed product to human gastric epithelial cells.** To test whether the 20K cloned product, which was shown to bind to NL residues in fetuin, also binds to a natural receptor present on gastric epithelial cells, the following experiments were performed. Antral biopsies were obtained from a volunteer who did not have an *H. pylori* infection, as confirmed by serology, a [$^{13}$C]urea breath test, histology, and culture. Serial tissue sections were exposed to total cell proteins from *E. coli* carrying pHPA24 (expressing ORF2) or pHPA23, to total cell proteins from *H. pylori* or purified 20K adhesin (positive controls), or to purified *H. pylori* urease or total cell proteins from *E. coli* carrying pBluescript without an insertion (negative controls). In addition, NL was added to a second set of samples; after 1 h at room temperature, the tissue sections were washed exhaustively with PBS containing an increased concentration of salt to discourage nonspecific hydrophobic binding. The tissue sections exposed to samples that were pretreated with NL were washed with the same buffer containing NL. After additional washes with PBS alone, the samples were treated with anti-NLBH. Adherence of proteins to the tissue was visualized by using a secondary antibody conjugated to alkaline phosphatase and the substrate containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

The product of pHPA24 (ORF2) bound specifically to the surface of gastric epithelial cells (Fig. 5A), as did the adhesin present in the *H. pylori* total protein preparation and the purified 20K adhesin (data not shown). The product of pHPA23 did not bind to the gastric epithelial cells (Fig. 5B). None of the negative controls, including purified urease, bound to the cells (data not shown). The specificity of the positive reaction was confirmed by the fact that no reaction was observed when only the primary or secondary antibodies were added to the tissue samples and by the fact that binding was blocked by NL.

These results are in very good agreement with the recent

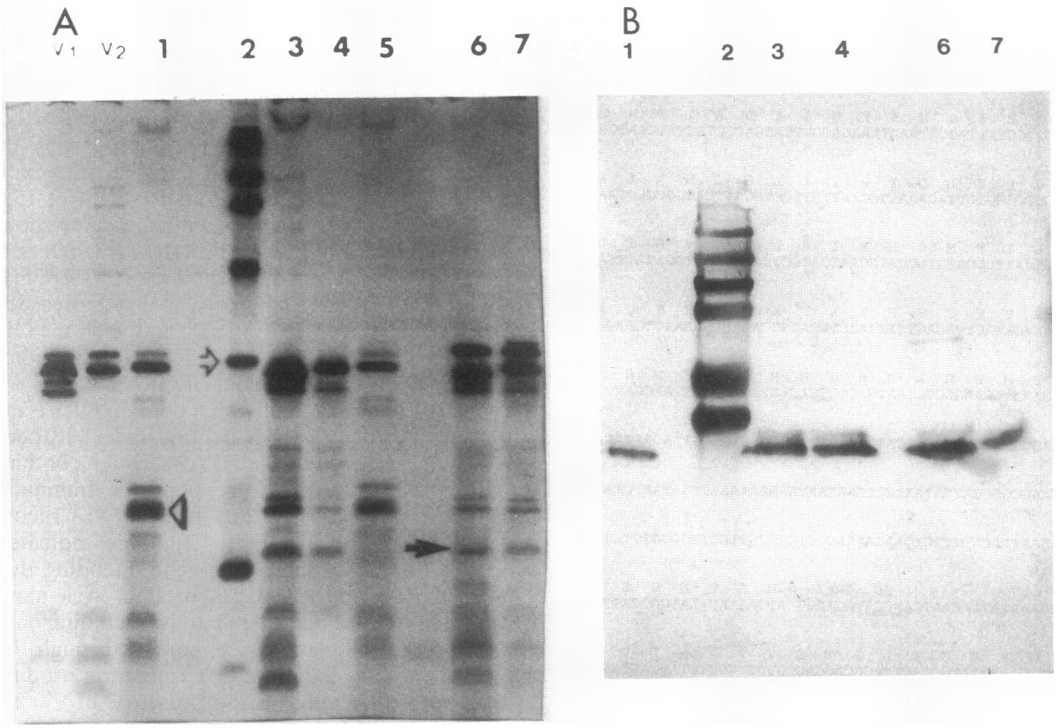


FIG. 4. Cloned polypeptides expressed by T7 polymerase, analyzed on 15% (A) and 12% (B) SDS–polyacrylamide gels. (A) Fluorography of polypeptides labeled with [$^{35}$S]methionine. Lanes 1 and 5, polypeptides encoded by pHPA24 in pBluescript; lane 2, $^{14}$C-labeled molecular weight standards (molecular weights, 200,000, 97,000, 69,000, 46,000, 30,000, and 14,000); lane 3, polypeptides encoded by pHPA in pBluescript; lane 6, polypeptides encoded by pHPA in PTZ18u; lane 4, polypeptides encoded by pHPA22 in pBluescript; lane 7, polypeptides encoded by pHPA22 in pTZ18u. The open arrow indicates the position of the 30K β-lactamase encoded by the vectors. Lane $V_1$ contained the peptides expressed by vector pTZ18u with no insert; lane $V_2$ contained the peptides expressed by vector pBluescript with no insert. The open triangle indicates the position of the 20K adhesin polypeptide. The solid arrow indicates the position of a 15K polypeptide encoded by ORF1. (B) Corresponding immunoblot. The samples and the positions of the samples were the same as for panel A except as follows: lane 4 contained the polypeptides encoded by pHPA24 in pBluescript, and lane 2 contained molecular weight standards (molecular weights, 180,000, 116,000, 84,000, 58,000, 49,000, 36,500, and 26,500). Reactions were with monospecific anti-*H. pylori* adhesin serum.

observations on identification of *N*-acetylneuraminyllactosyl ceramide as a gastric epithelial receptor for *H. pylori* (42) and provide additional evidence that the cloned 20K protein expressed by ORF2 has the same binding specificity as the NLBH.

**Identification of an amino acid sequence in HpaA corresponding to the *N*-acetylneuraminyl-2,3-lactose-binding motif of the *E. coli* SfaS adhesin.** Previously (14), we identified *N*-acetylneuraminyl-2,3-lactose as the receptor for the *H. pylori* fibrillar hemagglutinin. This same sialyl moiety was identified as the receptor for the fimbrial SfaS adhesin of *E. coli* (38). Recently, Morschhauser et al. (35) noted that the deduced amino acid sequence of SfaS includes a KARAVSK sequence and confirmed, by site-specific mutagenesis, that this sequence is part of the receptor-binding site of SfaS. The amino acid sequence KRTIQK in HpaA (amino acid residues 134 to 139 [Fig. 3]) corresponds to the sialic acid-binding sequence of SfaS, as well as to functionally identical sequences in *E. coli* K99 (24), in CFA/I (25) and in *Vibrio cholera* enterotoxin (30) (Table 3).

**Additional evidence that the product of the *hpaA* gene is the fibrillar hemagglutinin of *H. pylori*.** Attempts to perform an N-terminal amino acid analysis of the 20K adhesin purified from *H. pylori* failed apparently because the N terminus is blocked. However, in an attempt to further test whether the *hpaA* gene product is the NLBH adhesin produced by *H. pylori*, we reasoned that the receptor-binding site of the adhesin should be exposed to specific antibody, as well as to its sialic acid (NL) receptor. The specific antibody was obtained by using information contained in the cloned *hpaA* gene sequence; i.e., antibody was prepared against a synthetic peptide (LRPDPKRTIQKK) containing the amino acid sequence KRTIQK, which is the putative receptor-binding motif of HpaA (Fig. 3 and Table 3). A high-titer anti-peptide serum was obtained, and this serum did block hemagglutination of human erythrocytes by *H. pylori* cells at a 1:100 dilution, whereas preimmune serum from the same rabbit did not. Neither serum agglutinated the bacteria at this dilution. This confirmed that the LRPDPKRTIQKK sequence in the native molecule is exposed to antibody and that interference with this site blocked receptor-binding activity.

The specific anti-peptide antibody was purified by affinity chromatography and used for immunogold electron microscopy of glutaraldehyde-fixed but unstained *H. pylori* cells to localize the *hpaA* gene product. The results are shown in Fig. 6. Gold particles outlined an almost transparent material around *H. pylori*, as well as very small aggregates which were dislodged from the cells.

## DISCUSSION

We report here construction of a genomic *H. pylori* expression library. Using antibodies raised against the 20K

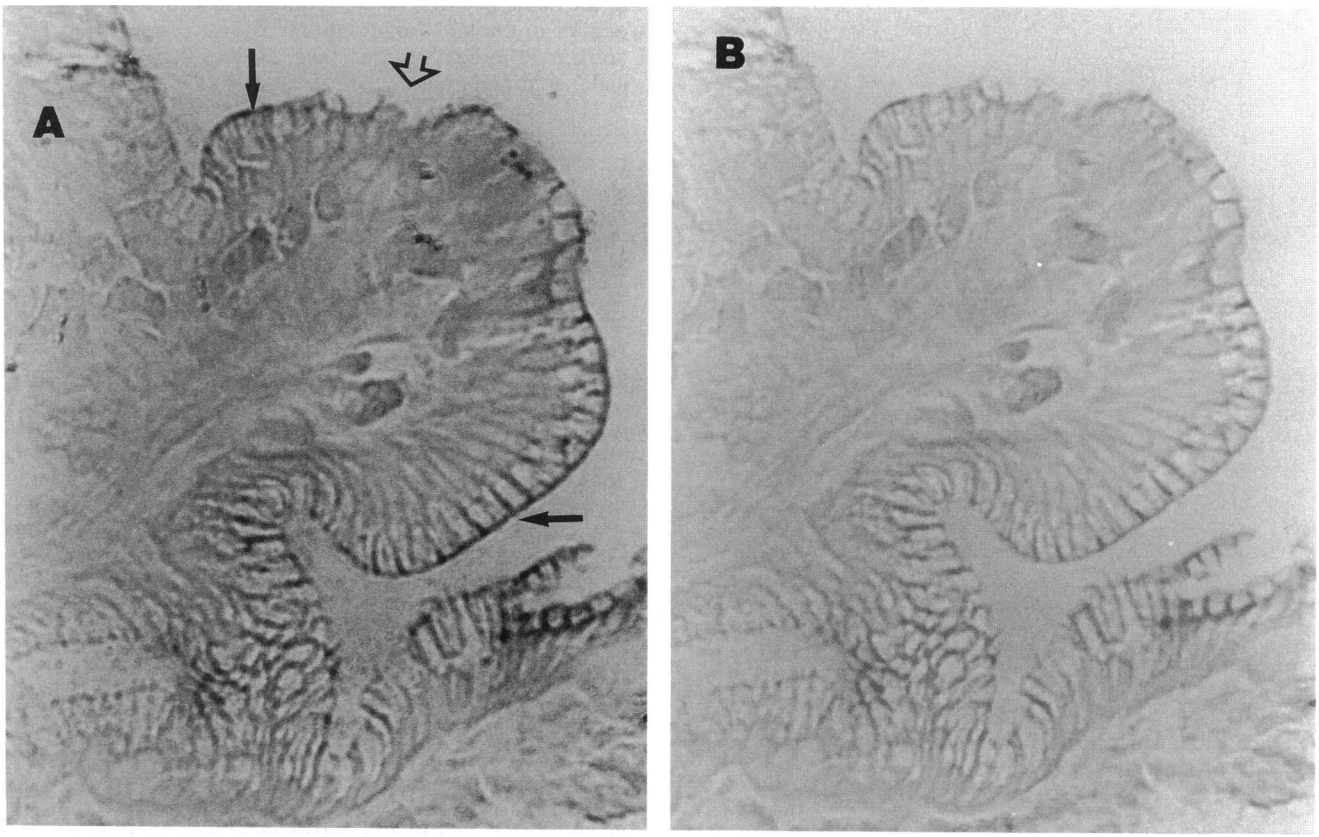


FIG. 5. Binding of the pHPA24-expressed product (ORF2) to human gastric epithelial cells. (A) Representative tissue section exhibiting an adherence-positive reaction obtained with the 20K protein expressed by pHPA24. A similar reaction was obtained with partially purified 20K *H. pylori* adhesin and with total *H. pylori* protein. Note that the ORF2 product (the 20K adhesin) bound only to intact epithelial cells (solid arrows) and not to cells damaged during tissue sectioning (open arrow). (B) Serial tissue section showing a representative adherence-negative reaction obtained with the pHPA23-encoded product. A similar negative reaction was obtained with purified *H. pylori* urease and tissue sections exposed to only anti-NLBH or secondary antibody.

adhesin, we identified a 1.4-kb DNA fragment, designated the adhesin region, that carries the genetic determinant for the 20K *H. pylori* adhesin.

A nucleotide sequence analysis revealed that the 1.4-kb DNA fragment contains three ORFs. ORF1 expresses a polypeptide with an estimated molecular weight of 15,000; the characteristics of the nucleotide sequence of ORF1 strongly suggest that the ORF1 product is a truncated peptide. Experiments are in progress to clone the entire ORF1 region. ORF2 expresses a 20K protein which has the same molecular weight, antigenic identity, and fetuin-binding specificity as the fibrillar NLBH of *H. pylori*. The same protein is also present in crude *H. pylori* cell lysates. Experiments not reported here have shown that a monoclonal antibody that reacts with the 20K adhesin purified from *H. pylori* also reacts with the ORF2 product. Thus, we have designated ORF2 *hpaA* and its product HpaA. There was no evidence of expression of ORF3.

TABLE 3. Comparison of receptor-binding motifs of *H. pylori* HpaA, three *E. coli* sialic acid-specific adhesins, and sialic acid-binding subunit B of *V. cholerae* heat-labile enterotoxin

| Sialic acid-binding protein | Amino acid sequence[a] |
|---|---|
| HpaA .....134-Lys | — Arg Thr Ile Gln Lys |
| SfaS .....116-Lys | Ala Arg Ala Val Ser Lys |
| K99 .....132-Lys | — Lys Asp — Asp Arg |
| CFA/I[b] ..... 56-Lys | — Lys Val Ile Val Lys |
| CT-B[c] ..... 62-Lys | — Lys Ala Ile Glu Arg |

[a] Arg and Lys are functionally similar amino acids, as are Thr and Ala, Ile and Val, and Gln and Asp. The number indicates the residue position in Fig. 3.

[b] CFA/I, colonization factor antigen I.

[c] CT-B, cholera toxin subunit B.

The nucleotide sequence of *hpaA* contains sequences (TTGACAA and TGTTAT) which resemble *E. coli* −35 and −10 promoter sequences (41) upstream from the ATG initiation codon; however, possible functions of these *H. pylori* sequences remain to be confirmed. The distance between these *H. pylori* promoterlike sequences is 29 bp, which is close to the 24 bp reported for similar sequences upstream from the cloned *H. pylori ureC* urease gene (26). Also, codons AUA, CUA, CGR, AGR, and GGR (where R is A or G), which might result in inefficient translation of *hpaA* in *E. coli* (19, 48), represent only 5% of the total codons in *H. pylori* DNA. According to Grosjean and Fiers (19), the preferential use of T in the third position rather than C is a characteristic of highly expressed genes in *E. coli*.

Two possible sites for a leader peptidase are found in the deduced amino acid sequence of HpaA. If the peptidase site

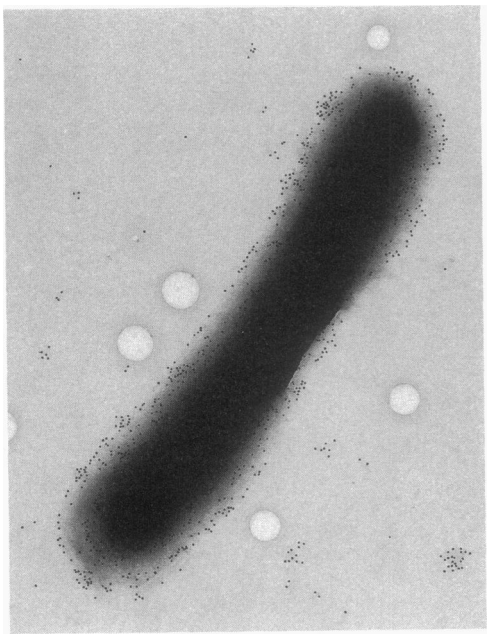

FIG. 6. Electron micrograph of an isolated unstained *H. pylori* cell that was exposed to antibody specific for a synthetic peptide which includes in its sequence the receptor-binding motif of HpaA and then anti-immunoglobulin G labeled with 10-nm-diameter gold particles. Magnification, ×23,000.

is at the phenylalanine residues, the putative leader sequence is similar in length and in the number of positively charged residues to the leader sequences of the *Bacteroides nodosus* FimB, FimC, and FimD proteins (21); if the peptidase site is at alanine (residue 11), the leader sequence is more similar to the leader sequences of PapA and 987P (2, 11). An N-terminal amino acid sequence analysis of the processed protein should resolve this question; unfortunately, numerous attempts at such an analysis have indicated only that the N terminus is blocked. Like many other fimbrial subunits (11, 40), HpaA has a penultimate aromatic amino acid residue.

Progress has been made in elucidating the molecular basis of sialic acid binding by bacterial adhesins. For example, amino acid residues Lys-132 and Arg-136 are important in the binding of the *E. coli* K99 fimbrial adhesin to its receptor, *N*-glycolylneuraminyllactose (23). Parkkinen et al. (38) showed that the *E. coli* fimbrial adhesin SfaS binds specifically to a terminal *N*-acetylneuraminyl-2,3-lactose moiety, which correlates with the receptor specificity of HpaA (14). Morschhauser et al. (35) noted that sialic acid-binding adhesin SfaS contains an amino acid sequence similar to that of K99; i.e., three of seven positions are occupied by either Lys or Arg. The results of site-specific mutagenesis experiments confirmed the importance of Lys-116 (homologous to Lys-132 of K99) and Arg-118 (homologous to Lys-133 of K99) in the receptor-binding activity of SfaS (35). Thus, it is highly significant that a homologous sialic acid-binding motif (KRTIQK) is found in HpaA. A similar motif also occurs in colonization factor antigen I of enterotoxigenic *E. coli* (25); furthermore, Ludwig et al. (30) and Finkelstein et al. (16) have identified a similar sialic acid-binding motif in *V. cholerae* cholera toxin subunit B which binds to Gm1. It is also notable that the HpaA sequence contains an Ile residue at the same position in the sequence at which an Ile residue occurs in cholera toxin subunit B and colonization factor antigen I, indicating that this residue also participates in sialic acid recognition.

The fact that affinity-purified antibody specific for a synthetic peptide containing the KRTIQK sequence blocked hemagglutination of human erythrocytes by *H. pylori* strongly supports the conclusion that this sequence represents the receptor-binding site of *H. pylori* NLBH and that HpaA codes for the adhesin. Also, by using immuno-gold electron microscopy, the peptide-specific antibody was used to localize HpaA on *H. pylori* cells, and the result was essentially the same as that obtained in previous work (14) in which NLBH-specific antibody reacted with the bacterial cells.

Our Western blot analysis clearly showed that the *H. pylori* adhesin can be expressed in *E. coli*. However, our attempt to agglutinate human erythrocytes with bacteria containing pHA24 failed. Thus, the 1.0-kb fragment contains the gene encoding the adhesin subunit but apparently not the genes required for the transport, assembly, and regulation of expression of functional fibrillae on the bacterial surface. Together, these results suggest that HpaA might have a genetic organization as complex as that of other fimbrial adhesins. Experiments to investigate this possibility are in progress.

## ACKNOWLEDGMENTS

We thank Magdalene So and Steve Moseley for their advice and encouragement and Sheila Hull for valuable help with the manuscript. We acknowledge the services of the Baylor College of Medicine Molecular Biology Information Resource Center.

This work was supported by U.S. Department of Agriculture grant 87-CRCR-1-2456 (to C.-H.L.), by Public Health Service grants DK-35369 (to D.G.E.) and DK-39919 (to D.Y.G.) from the National Institutes of Health, and by funding from the Department of Veterans Affairs (to D.J.E.).

## REFERENCES

1. **Andersen, L. P., S. Holck, L. Elsborg, and T. Justesen.** 1991. The *Helicobacter* (*Campylobacter*) *pylori*-colonized duodenal mucosa and gastric metaplasia. APMIS **99:**244–248.
2. **Baga, M., S. Normark, J. Hardy, P. O'Hanley, D. Lark, O. Olsson, G. Schoolnik, and S. Falkow.** 1984. Nucleotide sequence of the *papA* gene encoding the Pap pilus subunit of human uropathogenic *Escherichia coli*. J. Bacteriol. **157:**330–333.
3. **Bethesda Research Laboratories.** 1986. BRL pUC host: *E. coli* DH5alpha competent cells. Bethesda Res. Lab. Focus **8:**9.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Blaser, M. J.** 1990. Epidemiology and pathophysiology of *Campylobacter pylori* infections. Rev. Infect. Dis. **12:**S99–S106.
6. **Bode, G., P. Malfertheiner, and H. Ditschuneit.** 1988. Pathogenic implications of ultrastructural findings in *Campylobacter pylori* related gastroduodenal disease. Scand. J. Gastroenterol. **142:** 25–39.
7. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
8. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL-1Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
9. **Chung, C. T., S. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
10. **Correa, P., J. Fox, E. Fontham, B. Ruiz, Y. Lin, D. Zavala, N.**

**Taylor, D. Mackinley, E. de Lima, H. Portilla, and G. Zarama.** 1990. *Helicobacter pylori* and gastric carcinoma. Serum antibody prevalence in populations with contrasting cancer risks. Cancer **66:**2569–2574.

11. **De Graaf, F. K., and P. Klaasen.** 1987. Nucleotide sequence of the gene encoding the 987P fimbrial subunit of *Escherichia coli*. FEMS Microbiol. Lett. **42:**253–258.
12. **De Graaf, F. K., B. E. Krenn, and P. Klaasen.** 1984. Organization and expression of genes involved in the biosynthesis of K99 fimbriae. Infect. Immun. **43:**508–514.
13. **Evans, D. G., D. J. Evans, Jr., and D. Y. Graham.** 1989. Receptor-mediated adherence of *Campylobacter pylori* to mouse Y-1 adrenal cell monolayers. Infect. Immun. **57:**2272–2278.
14. **Evans, D. G., D. J. Evans, Jr., J. J. Moulds, and D. Y. Graham.** 1988. *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin of *Campylobacter pylori*: a putative colonization factor antigen. Infect. Immun. **56:**2896–2906.
15. **Evans, D. J., Jr., D. G. Evans, K. E. Smith, and D. Y. Graham.** 1989. Serum antibody responses to the *N*-acetylneuraminyllactose-binding hemagglutinin of *Campylobacter pylori*. Infect. Immun. **57:**664–667.
16. **Finkelstein, R. A., M. F. Burks, A. Zupan, W. S. Dallas, C. O. Jacob, and D. S. Ludwig.** 1987. Epitopes of the cholera family enterotoxins. Rev. Infect. Dis. **9:**544–561.
17. **Graham, D. Y., E. Adam, P. D. Klein, D. J. Evans, Jr., D. G. Evans, S. L. Hazell, L. C. Alpert, P. A. Michaletz, and H. H. Yoshimura.** 1989. Epidemiology of *Campylobacter pylori* infection. Gastroenterol. Clin. Biol. **13:**84B–89B.
18. **Graham, D. Y., P. D. Klein, D. J. Evans, Jr., D. G. Evans, L. C. Alpert, A. R. Opekun, and T. W. Boutton.** 1987. *Campylobacter pylori* detected noninvasively by the $^{13}$C-urea breath test. Lancet **ii:**1174–1178.
19. **Grosjean, C. S., and W. Fiers.** 1982. Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. Gene **18:**199–209.
20. **Hazell, S. L., A. Lee, L. Brady, and W. Hennessy.** 1986. *Campylobacter pyloridis* and gastritis: association with intercellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. **153:**658–663.
21. **Hobbs, M., B. P. Dalrymple, P. T. Cox, S. P. Livingstone, S. F. Delaney, and J. S. Mattick.** 1991. Organization of the fimbrial gene region of *Bacteroides nodosus*: class I and class II strains. Mol. Microbiol. **5:**543–560.
22. **Hui, W.-M., J. Ho, and S.-K. Lam.** 1991. Pathogenetic role of *Helicobacter pylori* in duodenal ulcer disease. Dig. Dis. Sci. **36:**424–430.
23. **Jacobs, A. A. C., B. H. Dimons, and F. K. deGraaf.** 1987. The role of lysine-132 and arginine-136 in the receptor-binding domain of the K99 fibrillar subunit. EMBO J. **6:**1805–1808.
24. **Jacobs, A. A. C., P. A. van den Berg, H. J. Bak, and F. K. De Graaf.** 1986. Localization of lysine residues in the receptor-binding domains of the K99 fibrillar subunit of enterotoxigenic *Escherichia coli*. Biochim. Biophys. Acta **872:**92–97.
25. **Karjalainen, T. K., D. G. Evans, M. So, and C.-H. Lee.** 1989. Molecular cloning and nucleotide sequence of the colonization factor antigen I of *Escherichia coli*. Infect. Immun. **57:**1126–1130.
26. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. J. Bacteriol. **173:**1920–1931.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
28. **Lingwood, C. A., H. Law, A. Pellizzari, P. Sherman, and B. Drumm.** 1989. Gastric glycerolipid as a receptor for *Campylobacter pylori*. Lancet **ii:**238–241.
29. **Loffeld, R. J. L. F., I. Willems, J. A. Flendrig, and J. W. Arends.** 1990. *Helicobacter pylori* and gastric carcinoma. Histopathology **17:**537–541.
30. **Ludwig, D. S., R. K. Holmes, and G. K. Schoolnik.** 1985. Chemical and immunological studies on the receptor binding domain of cholera toxin B subunit. J. Biol. Chem. **260:**12528–12534.
31. **Majewski, S. I. H., and C. S. Goodwin.** 1988. Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. J. Infect. Dis. **157:**465–471.
32. **Malfertheiner, P., G. Bode, A. Stanescu, and H. Ditschuneit.** 1989. Gastric metaplasia and *Campylobacter pylori* in duodenal ulcer disease: an ultrastructural analysis. Gastroenterol. Clin. Biol. **13:**71B–74B.
33. **Marshall, B. J.** 1990. *Campylobacter pylori*: its link to gastritis and peptic ulcer disease. Rev. Infect. Dis. **12:**S87–S93.
34. **Morris, A., and G. Nicholson.** 1987. Ingestion of *Campylobacter pyloridis* causes gastritis and raised fasting gastric pH. Am. J. Gastroenterol. **82:**192–199.
35. **Morschhauser, J., H. Hoschutzky, K. Jann, and J. Hacker.** 1990. Functional analysis of the sialic acid binding adhesin SfaS of pathogenic *Escherichia coli* by site-specific mutagenesis. Infect. Immun. **58:**2133–2138.
36. **Offerhaus, G. J. A., E. N. Molyvas, and P. J. Hoedemaeker.** 1990. *Helicobacter pylori* infection of gastric mucin cell metaplasia: the duodenum revisited. J. Pathol. **162:**239–243.
37. **Palva, E. T., T. R. Hirst, S. J. S. Hardy, J. Holmgren, and I. Randall.** 1981. Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli*. J. Bacteriol. **146:**325–330.
38. **Parkkinen, J., G. N. Rogers, T. Korhonen, W. Dahr, and J. Finne.** 1986. Identification of the O-linked sialyloligosaccharides of glycophorin A as the erythrocyte receptors for S-fimbriated *Escherichia coli*. Infect. Immun. **54:**37–42.
39. **Perez-Perez, G. I., D. N. Taylor, L. Bodhidatta, J. Wongsrichanalai, W. B. Baze, B. E. Dunn, P. D. Echeverria, and M. J. Blaser.** 1990. Seroprevalence of *Helicobacter pylori* infections in Thailand. J. Infect. Dis. **161:**1237–1241.
40. **Roosendaal, B., W. Gaastra, and F. K. de Graaf.** 1984. The nucleotide sequence of the gene encoding the K99 subunit of enterotoxigenic *Escherichia coli*. FEMS Microbiol. Lett. **22:**253–258.
41. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13:**319–353.
42. **Saitoh, T., H. Natomi, W. Zhao, K. Okuzumi, K. Sugano, M. Iwamori, and Y. Nagai.** 1991. Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining. FEBS Lett. **282:**385–387.
43. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
46. **Short, J. M., J. M. Fernandez, J. A. Sorge, and W. D. Huse.** 1988. Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. **15:**7583–7600.
47. **Slomiany, B. L., J. Piotrowski, A. Samanta, K. VanHorn, V. L. N. Murty, and A. Slomiany.** 1989. *Campylobacter pylori* colonization factor shows specificity for lactosylceramide sulfate and GM3 ganglioside. Biochem. Int. **19:**929–936.
48. **Sorensen, M. A., C. G. Kurland, and S. Pedersen.** 1989. Codon usage determines translation rates in *Escherichia coli*. J. Mol. Biol. **207:**365–377.
49. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
50. **Tatsuta, M., H. Ishikawa, H. Iishi, S. Okuda, and Y. Yokata.** 1990. Reduction of gastric ulcer recurrence after suppression of *Helicobacter pylori* by cefixime. Gut **31:**973–976.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
52. **Zhang, H., R. Scholl, J. Browse, and C. Somerville.** 1988. Double-stranded DNA sequencing as a choice for DNA sequencing. Nucleic Acids Res. **16:**1220–1226.