CLONING AND DNA SEQUENCE ANALYSIS OF A PLASMID FROM *HELICOBACTER PYLORI*

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN THE GRADUATE SCHOOL OF THE

TEXAS WOMAN'S UNIVERSITY

DEPARTMENT OF BIOLOGY

COLLEGE OF ARTS AND SCIENCES

BY

JENNIFER MINNIS, B.S.

DENTON, TEXAS

AUGUST, 1994

TEXAS WOMAN'S UNIVERSITY
DENTON, TEXAS

July 8, 1994
Date

To the Associate Vice President for Research and Dean of the Graduate School:

I am submitting herewith a dissertation written by Jennifer Minnis entitled "Cloning and DNA Sequence Analysis of a Plasmid from *Helicobacter pylori*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Molecular Biology.

Sarah A. McIntire
Dr. Sarah A. McIntire, Major Professor

We have read this dissertation and recommend its acceptance:

John E. Kneseke
Robert C. Benjamin
Richard Rudick
Nathaniel Mills
Ruth Schmidt
Department Chairperson

Accepted
Leslie M Thompson
Associate Vice President for Research
and Dean of the Graduate School

ABSTRACT

Cloning and DNA Sequence Analysis of a
Plasmid from *Helicobacter pylori*

JENNIFER MINNIS

August, 1994

Helicobacter pylori is a pathogenic bacterium that resides in the gastric epithelium of some humans and is widely regarded as a cause of type B gastritis and duodenal ulcer. Many bacterial species contain plasmid DNA which encodes virulence factors. Little is known about the plasmid DNAs found in some isolates of *H. pylori*. The purpose of this study was to clone a plasmid from *Helicobacter pylori* and to determine and analyze the DNA sequence.

A 3.5 kbp plasmid (pHPM180) was isolated from a strain of *H. pylori* (HPM180) obtained from a patient with inactive duodenal ulcer. The plasmid was successfully cloned in both orientations in the M13mp18 bacteriophage, and these recombinant phages were replicated in *E. coli* DH5αF'. The DNA sequence was determined using the dideoxy-ribonucleotide chain termination method. A computer analysis of the sequence was performed with DNASIS and PC-Gene software packages. Two open reading frames (ORFs) were identified. ORF1 translated to 463 amino acids and a putative polypeptide with a molecular weight of 54517. ORF2 translated to 240 amino acids with a molecular weight of 28142. Ribosome binding consensus sequences were identified upstream from both ORFs, and promoter consensus sequences were identified upstream from ORF1. A 232 base pair direct repeat was also identified in the DNA sequence of pHPM180.

Extensive DNA sequence homology was found between pHPM180 ORF1 and a 684

base pair *Hin*dIII fragment of a 7.2 kbp *H. pylori* plasmid. Additional sequence identity was found between 142 bases in a 200 base pair overlap of pHPM180 DNA and a segment of *H. pylori* pHPK255, a plasmid that encodes a Gram-positive type replication protein. The area of homology, however, was not contained within the ORF of pHPK255. A ribonuclease protection assay determined that ORF1 is transcribed in *H. pylori* HPM180.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Helicobacter pylori (formerly called *Campylobacter pyloridis/pylori*) is a Gram-negative, spiral shaped bacterium that colonizes human gastric mucosa. This microorganism is commonly found in patients with chronic gastritis and duodenal ulceration (21). The bacteria are 3 μm x 0.5 μm in size and have 1-6 polar flagella (21). *H. pylori* was initially discovered and successfully cultured in 1982 by a pathologist, Dr. Robin Warren, in Perth, Australia (31), who found *Helicobacter pylori* in biopsy samples of stomach tissue taken from patients with digestive complaints. He shared his findings with a colleague, Dr. Barry Marshall, who also found the microorganism in stomach biopsy samples from virtually every patient with stomach inflammation. The microorganism was initially described as a *Campylobacter*, but recent evidence (12) indicates that it is more closely related to *Wolinella* than to *Campylobacter*. Therefore, it was placed in a new genus, *Helicobacter*.

Initially, investigators were unsure as to whether *Helicobacter pylori* attached opportunistically to the already disrupted mucosa of persons with gastrointestinal disease, or if it was the cause of mucosal dysplasia in these individuals (14). Over the past decade, scientists have studied different aspects of this microorganism and have assembled compelling evidence that *H. pylori* is a causative agent of chronic active (type B) gastritis and is probably involved in causing most cases of duodenal ulcer. This is substantiated in a report by Armstrong *et al.* (1) where treatment of patients with bismuth and antibiotics effectively eliminated the bacteria as well as any associated gastritis. Furthermore, recent

review articles (3,17, 28) have compiled clinical data supporting the role of *Helicobacter pylori* in gastric disease. The current consensus of many clinical investigators is that some forms of gastritis and duodenal ulceration can be caused by *H. pylori* infection. Additionally, Talley *et al.* (26) reported a significant association between noncardiac gastric cancer and infection with *H. pylori*.

H. pylori has been known to survive asymptomatically in the gastrointestinal tract of patients for long periods of time and tends to be more prevalent among older individuals (13). Among possible virulence mechanisms associated with the bacterium are cellular adhesion factors (15) which could aid the bacterium in attachment to the gastric epithelium at intercellular junctions beneath the gastric mucosa. These adhesion factors would be important in initial colonization by the bacteria. Another potential virulence related protein from *H. pylori* is a 128 kDa protein encoded by chromosomal DNA that is associated with cytotoxin production (7). This protein produces vacuolization in cells grown in tissue culture. A cell surface-associated urease was purified and characterized (7, 9) and is thought to aid in initial colonization of the normally acid stomach by neutralizing the immediate environment around the bacterial cell. *H. pylori* also produces oxidase and catalase (17) which could enhance its survivability. An additional protein secreted by *H. pylori* is presumably a mucin protease (25) as it degrades mucin. Such a protease could render the epithelial layer of the stomach available for colonization by *H. pylori*. Finally, Dunn *et al.* (10) identified a water-extractable protein that is homologus to the chaperonin Cnp60 family of heat shock proteins, although its function in *H. pylori* has not been determined.

The mode of transmission of *H. pylori* is unknown. Since there tends to be

intrafamilial clustering of the microorganism, where spouses and children of patients infected with the bacteria are more often infected than control groups (18, 19), a person-to-person spread, perhaps by the fecal-oral route, seems likely. Interestingly, it has been observed that patients with duodenal ulceration and associated *H. pylori* infection have increased serum levels of pepsinogen I (6). Furthermore, it has been documented that an *H. pylori* protein can inhibit acid secretion by parietal cells in the stomach (4). These studies show that when the bacteria are eradicated, there is a subsequent decrease in serum pepsinogen I levels, and acid secretion returns to normal.

To date, the exact mechanism(s) of pathogenicity of *H. pylori* has not been elucidated. A full understanding of the nature of infection, the mechanisms of pathogenicity, and the means of eradication of the bacterium will necessitate a complete characterization of the organism including biochemical, molecular, and cellular approaches. Convenient animal models for studying pathogenicity of *H. pylori* are not available. Since many bacterial species contain plasmid DNAs which encode virulence factors such as enterotoxins, antibiotic resistance factors, or proteins involved in cell adhesion, a study of *H. pylori* plasmids may provide relevant information concerning such mechanisms in *H. pylori* infection. Therefore, the goal of this study was to characterize the physical structure and determine potential polypeptides encoded by a plasmid isolated from *H. pylori*.

Early studies of *H. pylori* plasmid DNA found these non-chromosomal DNAs ranged from 1.8 to 40 kilobase pairs (kbp) in size (24, 29), and were found in 58% of isolates. McIntire and Peterson (23), however, using a better isolation method, reported plasmids in 80% of clinical isolates. These plasmids ranged in size from 3.5-23 kbp. Restriction enzyme profiles of *H. pylori* plasmids (11, 22) differ among isolates from different patients but are the same within one individual. This information has been useful in following the

recurrence patterns of *H. pylori* in the same individual with gastritis or duodenal ulceration. Recently a 1.5 kbp plasmid was isolated and DNA sequence determined (16). An open reading frame was identified which encodes a 25 kDa protein similar to plasmid replication proteins found in Gram-positive bacteria that replicate by a "rolling circle" type mechanism. Since this small plasmid is basically a replicating circle of DNA, characterization of larger *H. pylori* plasmids could identify other proteins that might be important in understanding the physiology of *H. pylori*. Such structures also could provide information about the role of *H. pylori* in gastrointestinal pathophysiology.

RESEARCH OBJECTIVES

The ultimate goals of this research project were to determine the nucleotide sequence of a 3.5 kbp plasmid from *H. pylori*, to analyze the sequence, and to determine if any potential mRNAs were transcribed in *H. pylori*. These goals were accomplished by:

1. purification of plasmid DNA (pHPM180) from an *H. pylori* strain (HPM180) known to contain a plasmid of approximately 3.5 kb;
2. restriction enzyme analysis of the plasmid to obtain a linearized form of the entire plasmid which could be ligated to the multicloning site of the M13mp18 vector;
3. ligation of pHPM180 DNA into the M13 vector DNA and transformation into *E. coli* DH5αF';
4. determination of the DNA sequence of both strands of pHPM180 using the Sanger dideoxyribonucleotide chain termination method;
5. analysis of the DNA sequence for open reading frames (ORFs) and regions of homology to known nucleic acid and amino acid sequences;

6. determination of transcription of ORFs in *H. pylori* using a ribonuclease protection assay (RPA).

CHAPTER II

MATERIALS AND METHODS

Materials

Bacterial, plasmid, and phage strains. *H. pylori* strain HPM180 was originally obtained from a gastric biopsy of a patient with inactive duodenal ulcer at the VA Medical Center in Dallas. A frozen culture of this strain in trypticase soy broth containing 25% glycerol and 10% horse serum was provided by Dr. S. McIntire. HPM180 was cultured at 37°C under microaerophilic conditions (5-12% CO_2) on brain heart infusion (BHI) medium supplemented with 10% horse serum.

E. coli DH5αF' is *supE*44 Δ*lacU*169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 and contains F' *proAB*. This strain was obtained from Dr. J. Knesek. It was cultured and maintained at 37°C on NZY agar medium.

M13mp18 vector is a modification of the F-specific filamentous bacteriophage M13. It contains the N-terminal portion of the *lacZ* (β-galactosidase) gene and can be used for α-complementation in a suitable *E. coli* host that lacks that portion of the gene (such as DH5αF'). The vector contains a multiple cloning site within the α-peptide region so that color selection of recombinant plaques can be used (colorless plaques contain insert, blue plaques do not contain insert). This vector was obtained from Gibco BRL.

Primers. The primer used for sequencing the first ~300 nucleotides of both strands of pHPM180 was the M13/pUC forward sequencing primer provided in the Sequenase kit (see commercial kits). Subsequent primers were 17, 19, or 20mer oligonucleotides with sequences derived from the ends of previously sequenced fragments of pHPM180 DNA.

These primers were purchased from Bio-Synthesis Inc. in Lewisville, Tx.

Enzymes. Enzymes utilized were T4 DNA ligase (USB), lysozyme (Sigma), shrimp alkaline phosphatase (USB), RNase A (Sigma), T7 RNA polymerase (Ambion), Sequenase Version 2.0 DNA polymerase (USB), Ambion proteinase K (2 mg/ml), and restriction endonucleases (Promega). Appropriate restriction enzyme buffers recommended by the supplier were utilized with the restriction enzymes.

Commercial kits. The following commercial kits were used: Elu-Quik DNA Purification System (Schleicher & Schuell), Magic Maxipreps DNA Purification System (Promega), Super Comp media and TXN salts (Bio101), Sequenase Version 2.0 DNA Sequencing Kit (USB), Nick Translation Reagent Kit (BRL), MAXIscript in Vitro Transcription Kit (Ambion), and RPA II Ribonuclease Protection Assay Kit (Ambion). These kits were used as specified by the manufacturers.

Media. *E. coli* strains were grown in NZY medium (amine, yeast extract, magnesium sulfate, and sodium chloride) purchased from Gibco BRL, or YT medium (tryptone, yeast extract, and sodium chloride) purchased from Bio101. The *H. pylori* strain was grown in brain heart infusion (BHI) medium purchased from Difco and supplemented with 10% horse serum (Sigma). These media were prepared according to the manufacturers' recommendations. The medium used for preparation of competent cells was Super Comp media (Bio 101).

Buffers and other reagents. Buffers used were TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0), TE (10 mM Tris, pH 7.5, 1 mM Na_2EDTA), Buffer I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), Buffer II (0.2 N NaOH, 1% SDS), Buffer III (3 M potassium acetate, 1.8 M formic acid, pH 4.8), Wash Buffer (0.1 M

potassium acetate, 50 mM MOPS, pH 8.0), LiCl Solution (10 M LiCl, 50 mM Tris-HCl, pH 8.0), Solution A (50 mM Tris-HCl, pH 8.0, 4% Triton X-100, 2.5 M LiCl, 62.5 mM EDTA), 5X Ligation Buffer (439 mM Tris-HCl, pH 7.6, 43 mM $MgCl_2$, 66 mM DTT, 439 mM ATP, 133 mg/ml PEG, 33 ug/ml BSA), PEG solution (20% PEG-8000, 3.5 M ammonium acetate), 1X TXN Salts (Bio 101), Lysis Solution (0.8% SDS, 5 mM Tris-HCl, 0.5 mM EDTA, pH 8.0, 6.6 mg/ml proteinase K), 10X SSC (1.5 M NaCl, 0.17 M sodium citrate, pH 7.0), Rapid Hybridization Solution (Stratagene), and guanidinium mixture (4 M guanidine thiocyanate, 0.1 M Tris-HCl, pH 7.5, 1% β-mercaptoethanol).

Other reagent grade materials used were 100%, 95%, 80%, and 70% ethanol, 1 M Tris-HCl, pH 8.0, 0.5 M EDTA, pH 8.0, 1.5 M and 3 M sodium acetate, pH 5.2, 1 mg/ml ethidium bromide, Tris-HCl buffered phenol/chloroform (1:1), phenol/chloroform/IAA (Ambion), 3 M sodium acetate, methanol, acetic acid, agarose, 10% X-Gal, 400 mM IPTG, isopropanol, 7.5 M and 5 M ammonium acetate, acryl/bis (19:1) solution (Amresco), TEMED (Amresco), ammonium persulfate (Bio-Rad), [^{35}S]-dATP (NEN), [α^{32}P]-UTP (Bio-Rad), 5X Ficol dye (0.1 M EDTA, 25% Ficol, 0.1% bromophenol blue, 0.1% xylene cyanol), Sephadex G-25 Nap-5 columns (Pharmacia), 10 M NaOH, 10% SDS, ultrapure urea, cesium chloride, 1-butanol, Safety-Solve scintillation coctail (RPi) and sucrose. The molecular size standard used was 1 kb DNA ladder (BRL) which contains DNA fragments of 12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 517; 506; 396; 344; 298; 220; 201; 154; 134; and 75 base pairs (bp).

Methods

For total RNA preparations from HPM180, CsCl centrifugations were performed in a Beckman model L5-65 ultracentrifuge with a SW-65 rotor. All other centrifugations utilized a Beckman JS-HS centrifuge with either a JS-7.5 swinging bucket rotor (for 15 and 50 ml tubes) or a JA-18.1 fixed angle rotor (for 1.5 ml microfuge tubes).

Plasmid preparation. Plasmid DNA (pHPM180) was purified from *H. pylori* isolate HPM180 using a modification of the alkaline/SDS extraction procedure of Birnboim and Doly (2). This modified procedure, developed by S. McIntire, increased the lysozyme concentration of the preparation to 30 μg/ml, and added a LiCl RNA precipitation step.

Sixteen 125 ml tissue culture flasks containing 25 ml each of BHI + 10% horse serum were inoculated with *H. pylori* strain HPM180. Cultures were grown at 37°C and 10% CO_2 for four days. Cells were transferred to eight 50 ml conical tubes and centrifuged at 5,000 rpm and 4°C for 10 min. Supernatants were discarded. Pelleted cells were resuspended in a total volume of 16 ml TE and dispersed into sixteen 1.5 ml microfuge tubes. Tubes were microfuged for 1 min and the supernatants were discarded. Each pellet was suspended in 50 μl of Buffer I by vigorous vortexing, and the tubes were placed on ice. Fifty microliters of Buffer I containing 20 mg/ml of lysozyme was added, and tubes were flicked to mix, and put on ice for 10 min. Two hundred microliters of room temperature Buffer II was added to the tubes which were mixed gently by inversion and incubated on ice for 15 min. One hundred and fifty microliters of room temperature Buffer III was added. The tubes were mixed gently by inversion, incubated on ice for 15 min, and microfuged 10 min. Supernatants were transferred to new tubes, and DNA was precipitated by adding 1 ml of ice-cold 95% ethanol. Tubes were incubated at -20°C for 20 min and centrifuged at 9,000 rpm and 4°C for 10 min. Supernatants were carefully

removed and discarded. Pellets were suspended in 100 µl of Wash Buffer, and 200 µl of ice-cold 95% ethanol was added. Tubes were placed at -20°C overnight. The next day, tubes were centrifuged at 9,000 rpm and 4°C for 15 min. Supernatants were removed and discarded. Pellets were suspended in 100 µl of Wash Buffer, and 200 µl of ice-cold 95% ethanol was added. Tubes were incubated at -20°C for 20 min. Pellets were suspended in 100 µl ultrapure water by vortexing, and 100 µl of LiCl solution was added. Tubes were incubated for 30 min on ice, and centrifuged at 9,000 rpm and 4°C for 15 min. Two supernatants were transferred to one new tube (8 tubes total). Three molar sodium acetate pH 5.2 was added to the tubes to obtain a final concentration of 0.1 M, and two volumes of ice-cold 95% ethanol were added (i.e; 14 µl of sodium acetate and 800 µl of ethanol to 400 µl of supernatant). Tubes were incubated at -20°C for 20 min, then centrifuged at 9,000 rpm and 4°C for 10 min, and supernatants were discarded. Pellets were suspended in 50 µl Wash Buffer, and two tubes were combined to one (4 tubes total). Two volumes of 95% ethanol were added to the tubes. Tubes were incubated for 20 min at -20°C and then centrifuged at 9,000 rpm and 4°C for 15 min. Pellets were washed once with 100 µl of ice-cold 70% ethanol and vacuum dried. Each pellet of plasmid DNA was suspended in 20 µl of ultrapure water (80 µl total).

To eliminate any residual RNA from the plasmid DNA obtained from the above procedure, the S&S Elu-Quik DNA Purification Kit was used according to the manufacturer's instructions. The double stranded replicative form (RF) of M13mp18 (vector), and recombinant plasmids pMJM180a and pMJM180b were purified using the Magic Maxipreps DNA Purification System according to manufacturer's specifications.

Restriction enzyme analysis. Restriction enzymes corresponding to sites found in the multicloning site of M13mp18 were tested to determine whether any digested pHPM180 only once. Digestions were carried out by incubating 100 ng of plasmid DNA with 6-12 units of each restriction enzyme and 2 µl of 10X restriction buffer in a total volume of 20 µl for 1 hr at 37°C. Digested DNA was applied to a 0.7% agarose gel. Electrophoresis was carried out at 15 V/cm for 30 min in TBE buffer containing 0.25 µg/ml ethidium bromide. The gel was visualized on a long wave UV transilluminator (302 nm) and photographed using type 57 film in a Polaroid MP-3 camera equipped with an orange #15 filter.

In preparation for ligation of vector DNA (M13mp18) to insert DNA (pHPM180), 5 µg of the RF of M13mp18 was digested with 12 units of *Eco*RI, 5 µl 10X restriction enzyme buffer, and 10 units of shrimp alkaline phosphatase (SAP) in a total volume of 50 µl for 60 min at 37°C. This digestion produced linearized vector that could not religate to itself because the 5' phosphates had been removed. Insert DNA was digested as follows: 600 ng of pHPM180 was combined with 12 units of the above restriction enzyme and 2 µl of 10X restriction buffer in a total volume of 20 µl and incubated as above. The digestion resulted in linearized insert DNA with ends compatible to the vector.

The restriction enzyme and SAP were inactivated by incubating each digest for 15 min at 65°C, extracting once with an equal volume of phenol/chloroform (1:1), and centrifuging at 12,000 rpm and 4°C for 5 min. The aqueous phase was removed and placed in a clean tube. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. Tubes were placed at -70°C for 30 min, then centrifuged at 9,000 rpm and 20°C for 20 min. Pellets were washed twice with 70% ethanol and vacuum dried. The vector DNA pellet was dissolved in 100 µl of ultrapure water (final

concentration of vector DNA ~ 50 ng/μl), and the insert DNA pellet was dissolved in 40 μl ultrapure water (final concentration of insert DNA ~ 15 ng/μl).

Ligation reaction. The ligation mixture contained 100 ng digested vector DNA, an equimolar amount of insert DNA (60 ng), 4 μl of 5X ligation buffer, 1 unit T4 DNA ligase, and ultrapure water to a total volume of 20 μl in a 1.5 ml microfuge tube. The mixture was incubated at 20°C for 2 h and then stored at -20°C until ready for transformation reactions.

Competent cells and transformation. *E. coli* DH5αF' was grown in 50 ml Super Comp media at 37°C with shaking to an $OD_{600\ nm}$ of 0.6. The bacterial cells were made competent for transformation by placing 25 ml of the suspension into a 50 ml conical tube and centrifuging at 6,000 rpm and 4°C for 15 min. The pellet was suspended in 15 ml 1X TXN salts and incubated on ice for 30 min. After incubation, the ice cold mixture was centrifuged at 6,000 rpm and 4°C for 15 min. The resulting pellet was suspended in 5 ml 1X TXN and was ready for transformation. For infection of DH5αF' with recombinant phage DNA, 200 μl of competent cells was added to 20 μl of the ligation reaction and incubated on ice for 30 min. The mixture was heat shocked for 1 min at 42°C.

Screening for clones. Phage infected cells were diluted by pipetting 5, 10, 25, 50, and 100 μl of cells into five 17 x 100 mm glass tubes containing 1 ml of NZY medium. To these dilutions was added 1 ml of freshly grown DH5αF' ($OD_{600\ nm}$ = 0.8), 10 μl 10% X-Gal, and 5 μl 400 mM IPTG. Five 13 x 100 mm glass tubes, each containing 2 ml of 1.6% agarose and 1 ml of 4X NZY, were placed into a 58°C heating block. The solution from the heated tubes was mixed gently with the tubes containing transformed cells and then poured over five NZY plates. These plates were allowed to solidify for 15 min at room

temperature and then incubated at 37°C overnight. The next day, all well-separated colorless plaques that formed were picked by stabbing the tip of a sterile Pasteur pipette into the agar. Plugs were placed into separate 2 ml aliquots of NZY medium, and incubated at 37°C for 2 h to allow phage particles to move into the media from the agar plugs. The phage solutions were centrifuged, and supernatants were removed to new tubes to be used as stock phage solutions.

Plaque purification of phage stocks was accomplished by serially diluting the stocks into 1 ml of NZY medium in 13 x 100 mm glass tubes to obtain dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} for each stock. These dilutions were treated as previously described for the transformed cells and poured over NZY plates. A well-separated colorless plaque of each clone was picked, and a mini-preparation was made of RF DNA. Each plaque was picked and incubated in 1 ml of NZY medium for 2 h at room temperature. Next, 200 µl of the phage suspension was added along with 100 µl freshly grown DH5αF' ($OD_{600\ nm}$ = 0.8) to 4 ml NZY medium in a 15 ml polypropylene tube and incubated for 5 h at 37°C with shaking. The bacterial suspension was centrifuged at 4°C and 9,000 rpm for 5 min. Resulting supernatants were removed and stored at 4°C as plaque purified phage stocks for later preparation of single-stranded DNA. The remaining pellets were suspended in 200 µl of Solution A and 200 µl of Tris-HCl buffered phenol/chloroform (1:1). After vortexing, the mixture was microfuged for 5 min. Supernatants were removed, and pellets were washed with 70% ethanol and vacuum dried. To complete the RF preparation, dry pellets were dissolved in 10 µl of ultrapure water and 1 µl of 1 mg/ml RNase A and then incubated at room temperature for 5 min. The RF preparations were screened to determine if any contained the entire pHPM180

plasmid by digesting with *Eco*RI and analyzing the digests on a 0.7% agarose gel, as previously described. One clone (14s), showing fragments at the same location in the gel as vector and insert DNA controls, was used to derive recombinants with the insert in both orientations. Clone 14s DNA was digested with *Eco*RI, religated, and transformed as above to obtain clones pMJM180a and pMJM180b with pHPM180 DNA in opposite orientations. Plaque purified phage stocks prepared from pMJM180a and pMJM180b were used for preparation of single-stranded DNA for sequencing.

Restriction enzyme analysis of pMJM clones. M13mp18 RF, pHPM180, pMJM180a, and pMJM180b DNAs were digested with *Eco*RI, *Dra*I, and *Eco*RI/*Dra*I double digests by incubating ~100 ng of DNA, 12 units of restriction enzyme, 2 µl of restriction enzyme buffer, and ultrapure water to 20 µl in 1.5 ml microfuge tubes for 1 hr at 37°C. Following addition of 4 µl of 5X Ficol dye, electrophoresis was performed on a 1% (w/v) agarose gel at 15 V/cm for 1.5 hr. The gel was visualized and photographed as previously described.

Southern blot analysis of pMJM clones. Clones pMJM180a and pMJM180b were digested with *Eco*RI and separated on a 0.7% (w/v) agarose gel as previously described. Fragments of ~3.5 kbp for each clone were eluted from the gel using the procedure recommended in the S&S Elu-Quik kit. The purified DNA fragments were labeled with [^{35}S]-dATP using the BRL Nick Translation Reagent kit according to the manufacturer's instructions. Unincorporated nucleotides were removed from probe samples by chromatography on Pharmacia Nap-5 (Sephadex G-25) columns as per the manufacturer's instructions. Counts per minute were determined by placing 2 µl of probe sample into 2 ml of Safety-Solve scintillation cocktail and counting in a Beckman LS 9000 liquid

scintillation counter. Probe A corresponded to the 3.5 kbp fragment of pMJM180a, and Probe B corresponded to the 3.5 kbp fragment of pMJM180b.

Samples of M13mp18 RF, pHPM180, pMJM180a, and pMJM180b DNA were digested with *Eco*RI and *Dra*I as previously described, and fragments were separated on a 1% (w/v) agarose gel. The gel was loaded with two samples of each digested DNA, such that identical banding patterns were obtained on each side for separate probing with probes A and B. The gel was incubated in 100 ml of 0.5 M NaOH, 1.5 M NaCl for 30 min to denature the DNA and then incubated in 100 ml of 1 M Tris-HCl, pH 8.0, 1.5 M NaCl to neutralize. DNA from the gel was transferred to a Duralon-UV membrane using a Stratagene Posi Blot pressure blotter and 10X SSC as specified by the manufacturer. After transfer of the DNA, the membrane was placed into a Stratagene UV Stratalinker, and the DNA was crosslinked to the membrane with 120,000 μJ of energy.

The membrane was rinsed with water and then divided down the center yielding two identical halves. Each half of the membrane was placed into a separate 50 ml conical tube marked A or B. Five ml of Stratagene Rapid Hybridization Solution was added to each tube, and the tubes were incubated at 68°C overnight with rotation. The next morning, Probes A and B were boiled for 2 min and then cooled on ice. After cooling, 1 X 10^6 cpm (400 μl) of Probe A was added to tube A, and the same amount of Probe B was added to tube B. The tubes were incubated overnight at 68°C with rotation. The next day, the membranes were briefly rinsed with 2X SSC, 0.1% SDS at 37°C to remove the hybridization solution. The membranes were washed two times for 10 min at 37°C with 2X SSC, 0.1% SDS and then rinsed briefly with 2X SSC to remove the SDS. The membranes were dried in an 80°C oven for 10 min and then autoradiographed using

Kodak XAR X-Ray film for 1 mo. The film was developed according to manufacturer's specifications.

Preparation of single-stranded DNA. Single stranded DNA was prepared from pMJM180a and pMJM180b plaque purified phage stocks as follows: 0.5 ml of plaque-purified phage stock was added to 2.5 ml of fresh DH5αF', freshly grown in 2X YT medium to $OD_{600\ nm}$ = 0.8. The bacterial/phage suspension was allowed to stand for 15 min at 37°C, diluted into 500 ml of 2X YT, and incubated with shaking at 37°C overnight. The next day, 20 ml of bacterial suspension was added to a 50 ml conical tube and centrifuged for 20 min at 7,500 rpm and 5°C. The supernatant was poured into a different 50 ml tube and centrifuged as before. Next, 16 ml of supernatant was carefully removed from the top of the tube to a new tube where 5 ml of PEG solution was added. The resulting solution was incubated overnight at 4°C. The following day, the solution was centrifuged as described above. The resulting pellet was suspended in 400 µl of ultrapure water, transferred to a 1.5 ml microfuge tube, and extracted with an equal volume of phenol. After microfuging for 5 min, the upper aqueous phase was removed to a new tube, and the phenol extraction repeated. The solution was then extracted three times with an equal volume of chloroform. To the final extract, an equal volume of 7.5 M ammonium acetate was added along with two volumes of 100% ethanol. The solution was incubated on ice for 15 min and then centrifuged 20 min at 4°C and 9,000 rpm. The resulting pellet was vacuum dried, then dissolved in 50 µl TE buffer, and stored at 4°C until ready for DNA sequence analysis.

Sequencing reactions. Nucleotide sequence determination of both single-stranded recombinant clones provided the sequence of both strands of pHPM180 and was accomplished using [^{35}S]-dATP and the Sequenase Version 2.0 kit. Initial priming

reactions utilized the universal primer provided in the kit. This primer, which corresponds to a sequence upstream of the multicloning site of M13mp18, provided sequence information into the cloned fragments. Subsequent primers were 17, 19, or 20 base oligonucleotides with sequences complementary to the end portions of the sequence previously determined; thus, the sequences of the two complementary recombinant clones were determined in an overlapping fashion. Sequencing reactions were analyzed on 6% polyacrylamide gels [mix 15 ml acryl/bis (19:1) solution, 20 ml 5X TBE, 48 g urea, and ultrapure water to a final volume of 100 ml. Filter and add 30 µl TEMED and 1 ml 10% ammonium persulfate] with electrophoresis at 2000 V in 1X TBE buffer in a Model # DASG-500-33 nucleic acid sequencing apparatus manufactured by CBS Scientific Co. Gels were washed in a solution of 15% methanol and 5% acetic acid, vacuum dried and autoradiographed using Kodak XAR X-ray film for 24 h. Films were developed according to manufacturer's specifications.

Analysis of DNA sequence. The DNA sequence of pHPM180 was analyzed for restriction sites, open reading frames, promoter and ribosomal binding consensus sequences, and transcription termination sequences by using the DNASIS computer program (Hitachi). Open reading frames determined by this program were compared to Genbank DNA and Swiss protein databases using PC/GENE computer software (Intellegenetics). Dr. Kosaku Uyeda (V.A. Medical Center, Dallas, Tx.) kindly assisted in this analysis.

Isolation of total RNA from HPM180. Eight 250 ml tissue culture flasks, containing 50 ml BHI + 10% horse serum, were inoculated with HPM180 and incubated at 37^{o}C and 10% CO_2 for four days. The cells were pelleted in eight 50 ml conical tubes at 3,400 rpm

and 4°C for 20 min. Next, 1 ml of guanidinium mixture was added to each of the pellets (except 0.5 ml for the eighth pellet), and the tubes were vortexed until the cells were lysed and the solution became clear. The solution was divided among three tubes, each containing 2.5 ml of solution. One gram of CsCl was added to each tube to obtain a final concentration of 2.3 M CsCl in the cell lysates. To three separate 5 ml polyallomer centrifuge tubes, 1.2 ml of 5.7 M CsCl, 0.01 M EDTA, pH 7.5 was added and 2.5 ml of cell lysate solution was layered on top. The tubes were filled with the guanidinium mixture and separated from cellular debris in an ultracentrifuge at 35,000 rpm and 20°C for 16 hr. Supernatants were discarded, and the tubes were inverted with plugs of Kimwipes for 5 min to allow excess solution to drain. Pellets were carefully rinsed with 100 µl of ice-cold 70% ethanol and then dissolved in 150 µl (50 µl each pellet) 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% SDS and combined into one 1.5 ml microfuge tube. The solution was extracted with 150 µl chloroform/1-butanol (4:1), and the upper aqueous phase was removed to a new microfuge tube. The organic phase was reextracted with 100 µl 10 mM Tris-HCl, 5 mM EDTA, and the aqueous phase was combined with that of the first extraction. One-tenth volume (28 µl) of 3 M sodium acetate and 2.2 volumes (550 µl) of ice-cold 100% ethanol were added to the extracts. The solution was incubated overnight at -20°C. The next day, the tube was centrifuged at 12,000 rpm and 4°C for 30 min. The pellet was washed with 100 µl ice-cold 70% ethanol and vacuum dried for 15 min. The dried pellet was dissolved in 60 µl of ultrapure water and stored at -80°C.

Preparation of antisense RNA probes. Preparations of antisense RNA probes to pHPM180 ORF1 transcripts, mouse β-actin control RNA, and preparation of RNA size markers were accomplished using the MAXIscript in Vitro Transcription Kit as described

by the manufacturer (Ambion). T7 RNA polymerase from the kit was used for all three reactions. The template for transcription of the ORF1 antisense probe was prepared by incubating 12 μg of pHPM376 DNA, 29 μl ultrapure water, and 2 μl RNase cocktail (Soln. R Ambion RPA II kit) in a 1.5 ml microfuge tube for 5 min at 37°C, and then vortexing the mixture. Fifty microliters of this treated DNA was linearized by digesting overnight at 37°C with 30 units of *Pvu*II and 20 μl of 10X restriction enzyme buffer in a total volume of 200 μl. Proteinase K (19 μl of 20 mg/ml) was added and incubation was continued at 50°C for 30 min. The solution was extracted once with 200 μl phenol/chloroform/IAA (Ambion), and the upper aqueous phase was removed to a new tube. Next, 20 μl 5 M ammonium acetate (RNase free) and 400 μl cold 100% ethanol were added, and the tube was placed for 30 min at -80°C. The solution was centrifuged at 12,000 rpm and 4°C for 20 min, and the pellet was washed with 200 μl of cold 70% ethanol and then vacuum dried for 15 min. The pellet was dissolved in 20 μl of RNase-free water and stored at 4°C as template A. The template for the β-actin probe and the RNA marker templates were provided by Ambion. Transcripts were labeled with [α^{32}P]-UTP using the Ambion MAXIscript kit. Unincorporated nucleotides were removed from probe samples, and counts per minute were determined as described under Southern blot analysis of clones section. RNA probes were stored at -80°C and used within 24 hr in the ribonuclease protection assay.

Ribonuclease protection assay. A ribonuclease protection assay was performed with the Ambion RPA II ribonuclease protection assay kit to determine if ORF1 was transcribed in HPM180 . Probes were used at 6 X 10^4 cpm. Following hybridization and treatment with RNase, the protected hybrids were separated on a 5% polyacrylamide/8 M

urea gel (14.4 g urea, 6 ml 5X TBE, 3.7 ml 40% acryl/bis (19:1), ultrapure water to 30 ml, filter, add 240 µl 10% ammonium persulfate and 16 µl TEMED) in 0.5X TBE at 15 V/cm for 2 hr. The gel was transferred to Whatman #3 chromatography paper, covered with plastic wrap, and exposed to X-Ray film for 24 hr. The film was developed according to the manufacturer's specifications.

Preparation of HPM180 total DNA. Two 250 ml tissue culture flasks containing 50 ml of BHI + 10% horse serum were inoculated with HPM180 and incubated at 37°C and 10% CO_2 for four days. The cells were centrifuged in two 50 ml conical tubes at 3,400 rpm and 4°C for 20 min. Pellets were suspended in a total volume of 6 ml TE and disbursed into six 1.5 ml microfuge tubes. Cells were pelleted in a microfuge for 1 min and supernatants were discarded. Each pellet was suspended in 200 µl ice-cold 50 mM Tris-HCl, pH 8.0, 25% sucrose. Next, 100 µl 30 mg/ml lysozyme in 0.25 M EDTA, pH 8.0 was added, and the tubes were incubated for 20 min on ice. Lysis solution (25 µl 5% SDS, 75 µl TE, and 50 µl 20 mg/ml proteinase K) was added to each tube. Tubes were mixed by inversion and incubated 60 min at 56°C. To each tube was added 23 µl 10 mg/ml RNase A, to give a final concentration of 50 µg/ml. Tubes were incubated 30 min at 37°C and then extracted two times with an equal volume of phenol, once with an equal volume of phenol/chloroform (1:1), and once with an equal volume of chloroform. Aqueous phases were pooled, and two volumes of ice-cold 100% ethanol and one tenth volume of 3 M sodium acetate were added. Tubes were incubated at -20°C overnight. The next day, the tubes were centrifuged at 9,000 rpm and 4°C for 30 min. Pellets were washed once with 70% ethanol and vacuum dried. Dried pellets were dissolved in 50 µl ultrapure water and stored at 4°C.

Southern blot analysis of total DNA. Approximately 200 ng of total cellular DNA and 100 ng of highly purified plasmid DNA from HPM180 were digested with *Dra*I as previously described. The digested DNAs as well as undigested DNA samples were separated by electrophoresis on a 0.7% (w/v) agarose gel at 15 V/cm for 40 min. The gel was irradiated with 60 mJ of energy in a Stratagene UV Stratalinker to enhance transfer of DNA from the gel to a Bio-Rad Zeta-Probe GT membrane. Transfer of DNA, prehybridization, and probing were accomplished as previously described for Southern blot analysis of clones, except that the probe was 5 X 10^6 cpm of the same RNA antisense probe to ORF1 transcripts as that used in the ribonuclease protection assay. The membrane was exposed to Kodak XAR-5 film and developed as previously described.

CHAPTER III

RESULTS

Purification of *H. pylori* plasmid DNA (pHPM180). Plasmid DNA from *H. pylori* strain HPM180 was purified using a modification of the alkaline/SDS extraction procedure of Birnboim and Doly (Fig. 1). Several bands were evident in the plasmid preparation, representing different conformers of the plasmid.

Restriction enzyme analysis of pHPM180 DNA. Purified plasmid (pHPM180) DNA was digested with restriction enzymes that occur in the phage cloning vector M13mp18 multicloning site to determine if any enzyme would digest the plasmid at a single site resulting in the linear form. Results shown in Fig. 2 indicated that *Eco*RI linearized the plasmid, producing a fragment of 3500 nucleotide base pairs. These results suggested that *Eco*RI would be a suitable enzyme for use in digestion of both the plasmid and vector DNAs in preparation for cloning experiments.

Cloning of the 3.5 kb plasmid in M13mp18. Both vector (M13mp18 RF) and plasmid (pHPM180) DNA were digested with *Eco*RI to obtain linearized forms. Additionally, vector DNA was incubated with shrimp alkaline phosphatase to decrease the probability of vector to vector ligation products and religation of vector DNA during the ligation reaction. The enzymes were inactivated by phenol/chloroform extraction, and linearized DNA was visualized under ultraviolet light on an agarose gel containing ethidium bromide (Fig. 3). Linearized plasmid and vector DNAs were ligated, and the mixture was used to transform competent *E. coli* DH5αF'. Forty-five colorless plaques were picked for screening by restriction enzyme analysis with *Eco*RI to determine clones containing

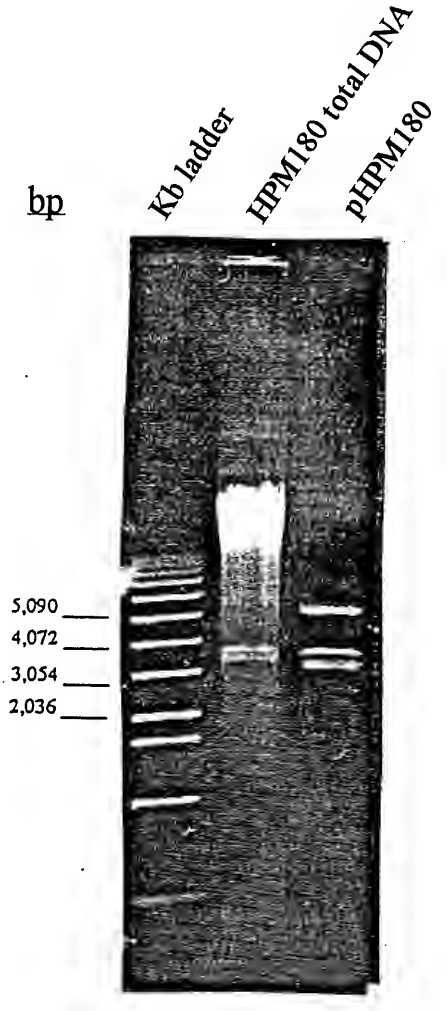


Fig. 1. Preparation of *H. pylori* DNA. O.7% (w/v) agarose gel showing total DNA and highly purified plasmid DNA (pHPM180) from *H. pylori* strain HPM180. The size in base pairs (bp) for the Kb ladder standard is indicated to the left of the figure.

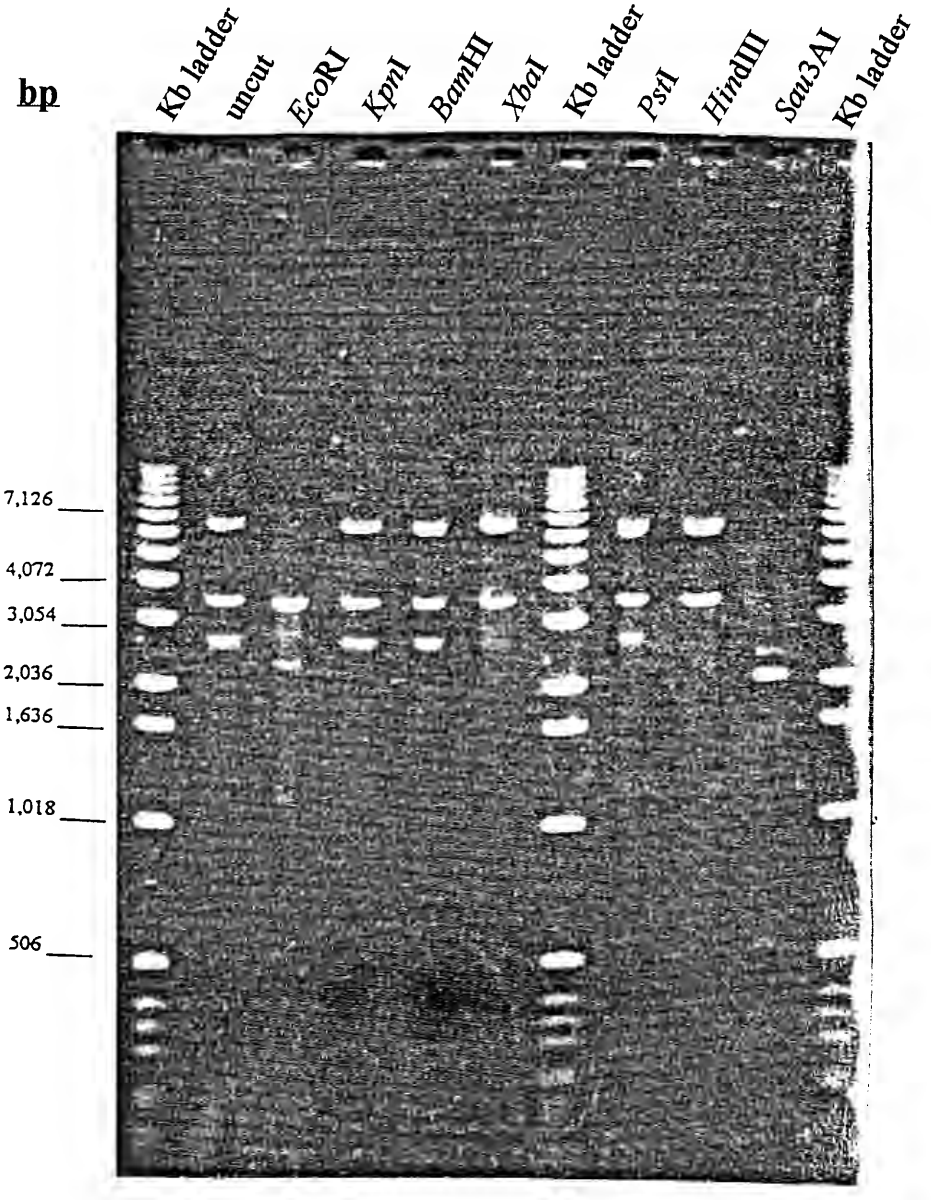


Fig. 2. Restriction enzyme analysis of pHPM180 DNA. 0.7% (w/v) agarose gel showing restriction enzyme analysis of pHPM180 DNA with enzymes occuring in the multicloning site of the phage vector M13mp18. The enzymes are indicated at the top of each lane. The size in base pairs (bp) for the Kb ladder standard is indicated to the left of the figure.

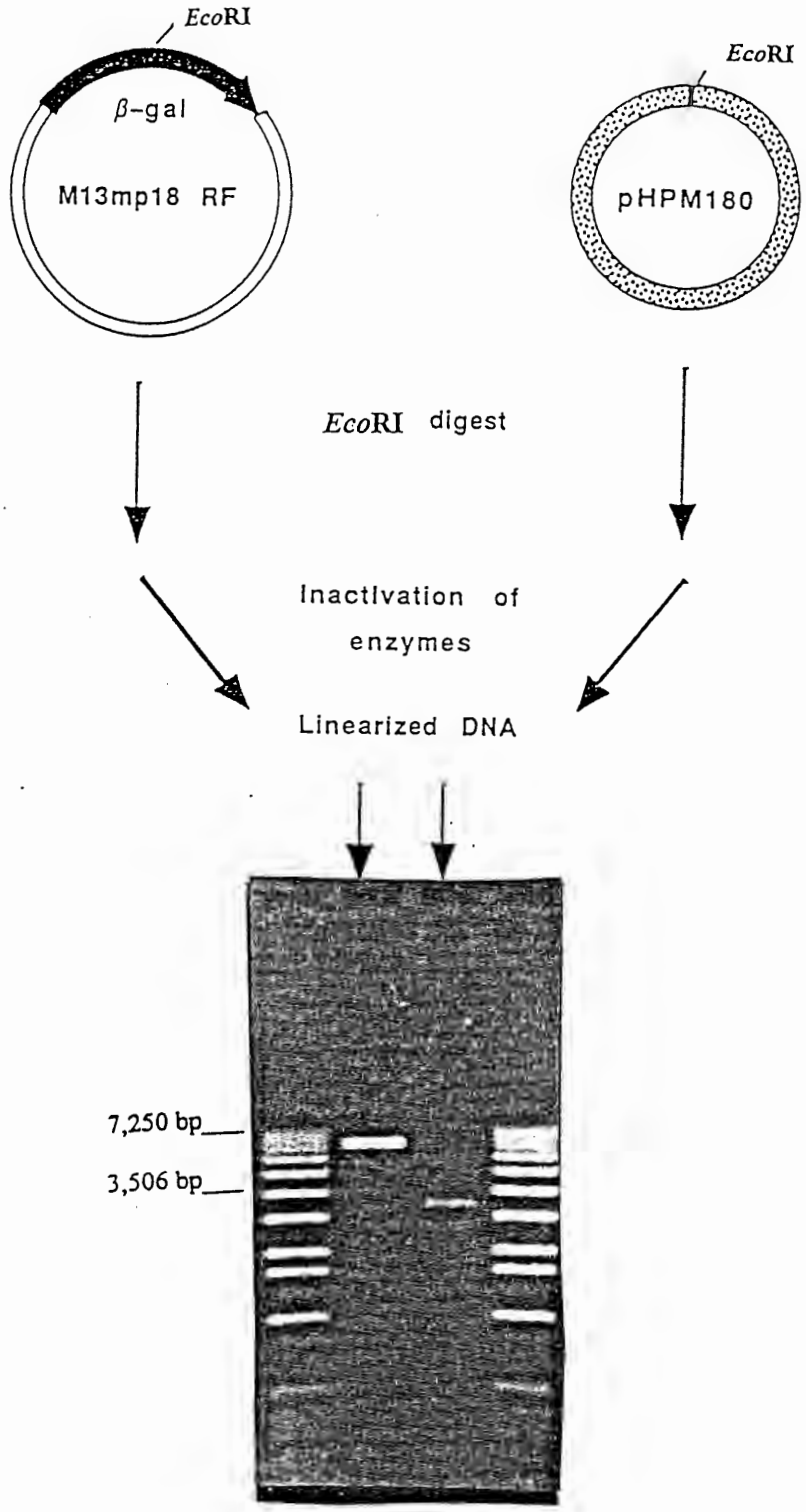


Fig. 3. Linearized vector and plasmid DNAs. 0.7% (w/v) agarose gel showing linearized vector (M13mp18) and pHPM180 DNA after digestion with *Eco*RI. Sizes in bp of the linearized plasmids are shown on the left.

pHPM180 DNA. One clone (14s), from the first twenty plaques screened, resulted in two fragments of expected size after digestion with *Eco*RI (Fig. 4). The sizes of these fragments corresponded to M13mp18 (7250 bp) and pHPM180 (~3500 bp).

Clone 14s DNA was digested with *Eco*RI and religated to obtain clones containing pHPM180 DNA in both orientations. Colorless plaques were picked and screened as before. Figure 5 illustrates expected and observed restriction fragments for pHPM180 inserts in opposite orientations. The *Eco*RI digest (Fig. 5B) showed that both pMJM180a and pMJM180b produced two fragments corresponding to the sizes of the vector RF (7.2 kbp) and pHPM180 DNA (3.5 kbp).

As illustrated in Fig. 5A, and shown in track 7 of Fig. 5B, *Dra*I digestion of pHPM180 DNA resulted in three fragments of 1.4, 1.2, and 0.9 kbp. Also, as shown in Fig. 5A and in track 6 of Fig. 5B, *Dra*I digestion of M13mp18 (RF) resulted in fragments of 4.2, 2.2, 0.36, 0.29, and 0.28 kbp (DNA map of M13mp18 courtesy of BRL).

Figure 5A illustrates an expected restriction map showing the composite *Dra*I fragments containing the *Eco*RI cloning sites for both the constructs. Figure 5B clearly showed the expected composite fragments. Track 8 contained fragments of 2.0 and 1.6 kbp and track 9 contained fragments of 3.0 and 0.6 kbp.

Further confirmation that pMJM180a and pMJM180b contained pHPM180 DNA was obtained by Southern blot analysis. The clones were digested with *Eco*RI, and fragments were separated on a 0.7% agarose gel. Fragments of 3.5 kbp were excised from the gel and used in a nick translation reaction to form two probes using [^{35}S]-dATP. Probe A was constructed using the 3.5 kbp fragment from pMJM180a as a template, and Probe B was constructed from the pMJM180b 3.5 kbp fragment. M13mp18 RF, pHPM180, pMJM180a, and pMJM180b DNAs were digested with *Eco*RI and *Dra*I enzymes, and

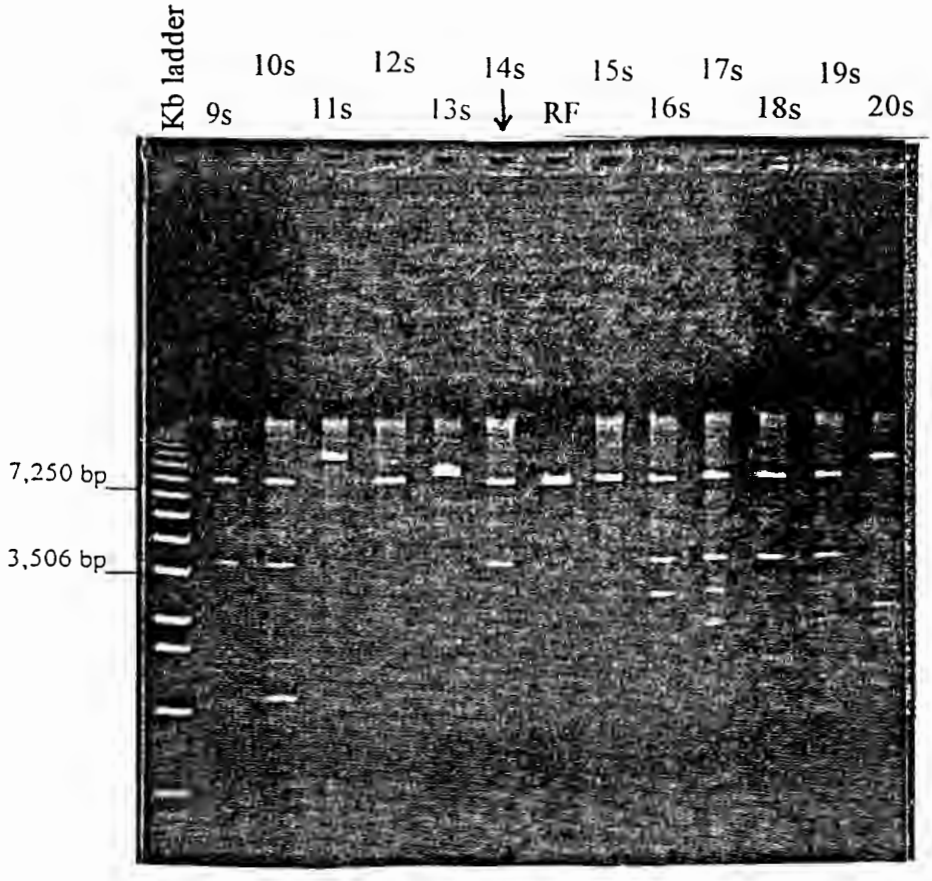


Fig. 4. Screening for clones containing pHPM180 DNA. 0.5% (w/v) agarose gel showing *Eco*RI digests of clones 9s through 20s screened for those containing pHPM180 DNA. RF is M13mp18 vector DNA. Sizes of the M13mp18 (RF) vector DNA and of expected pHPM180 DNA insert are indicated on the left.

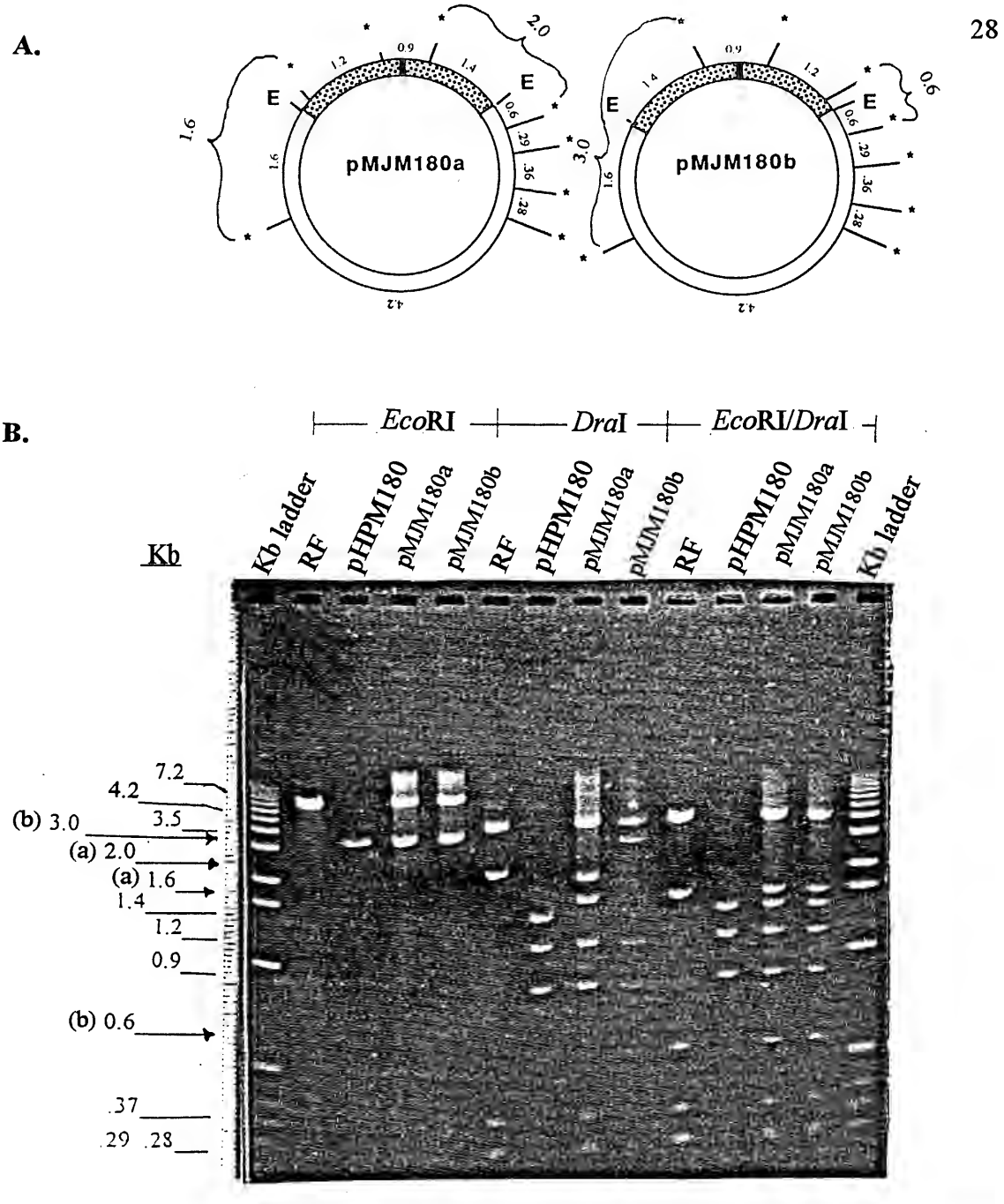


Fig. 5. Results of cloning experiments. Two clones, pMJM180a and pMJM180b, containing pHPM180 DNA in opposite orientations are shown.

A. Representative diagram of clones pMJM180a and pMJM180b showing expected *Eco*RI (E) and *Dra*I (*) restriction enzyme sites.

B. 0.5% (w/v) agarose gel showing *Eco*RI, *Dra*I, and *Eco*RI/*Dra*I digests of vector M13mp18 (RF), plasmid (pHPM180), and clone (pMJM180a and pMJM180b) DNAs. Composite fragments in the *Dra*I digest are indicated by arrows on the left of the figure.

duplicate digests were visualized on a 0.7% (w/v) agarose gel (Fig. 6A). DNAs were transferred from the gel to a nylon membrane which was subsequently divided to give two membranes with similar patterns of DNA samples. These membranes were probed separately with either Probe A or Probe B as described in METHODS. Fig. 6B showed that only fragments containing pHPM180 DNA hybridized to the probes.

Nucleotide sequence analysis. Initial nucleotide sequences of both pMJM180a and pMJM180b inserts were determined using universal M13 sequencing primers. Each sequencing reaction provided DNA sequence for approximately 300 bases. Subsequent 17, 19, or 20mer primers were chosen from a region near the end of each sequenced section of DNA (Fig. 7 and Table I). Primers were chosen based on having a 40-60% G-C content. In this manner, the DNA sequence of both strands of pHPM180 was determined (Fig. 8). The total sequence consisted of 3506 nucleotide base pairs. A 232 bp direct repeat (underlined in Fig. 8) interrupted by 35 bases of nonrepeating DNA was observed starting at nucleotide 1609.

Computer analysis of the nucleotide sequence revealed two open reading frames, ORF1 and ORF2 (Fig. 8). ORF1 began at nucleotide 2871 and was bisected by the unique *Eco*RI site. ORF2 began at nucleotide 896. Possible translation initiation codons for both ORFs were GUG. However, an AUG codon at position 908 could serve as an initiation codon for translation of ORF2. Boxes in Fig. 8 highlight the putative ribosome binding sequences (AGGAG and AGAAA respectively) located upstream from both ORFs, and putative -10 and -35 promoter sequences (TATTAT and TAGACA, respectively) upstream from ORF1. Single letter code for the derived amino acid sequence of ORF1 and ORF2 are indicated below the codons. ORF1 could encode a polypeptide of 463

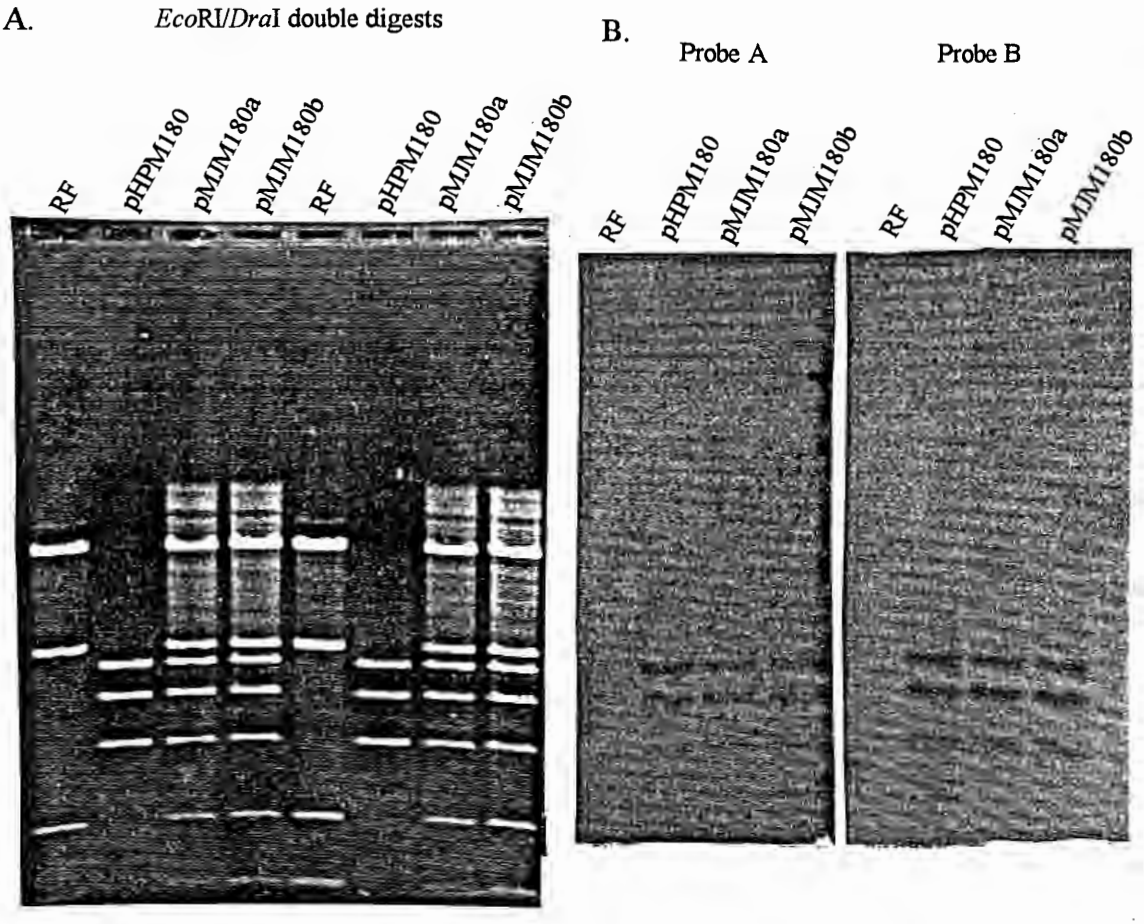


Fig. 6. Confirmation that pMJM clones contain *H. pylori* plasmid DNA.
A. 0.7% (w/v) agarose gel showing duplicate *Eco*RI/*Dra*I double digests of vector (RF), plasmid (pHPM180), and clone (pMJM180a and pMJM180b) DNA.
B. Autoradiogram showing hybridization of probes made from pMJM180a (Probe A) and pMJM180b (Probe B) DNA to DNA transfered from the gel in Fig. 6A to a Duralon-UV membrane.

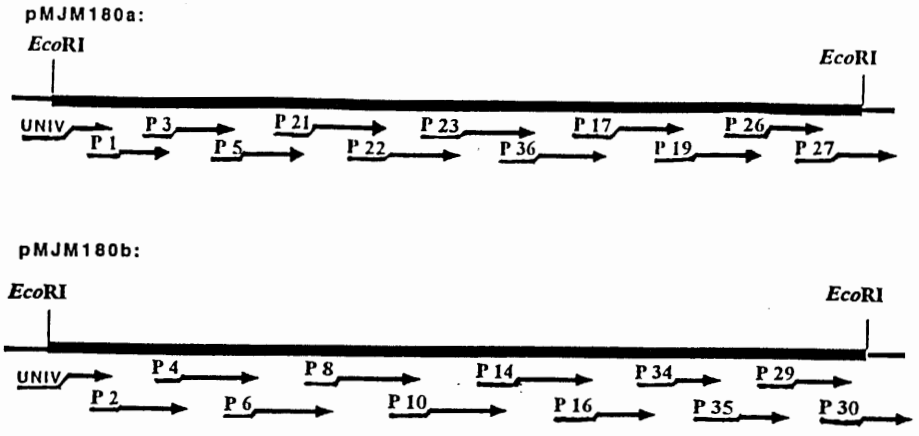
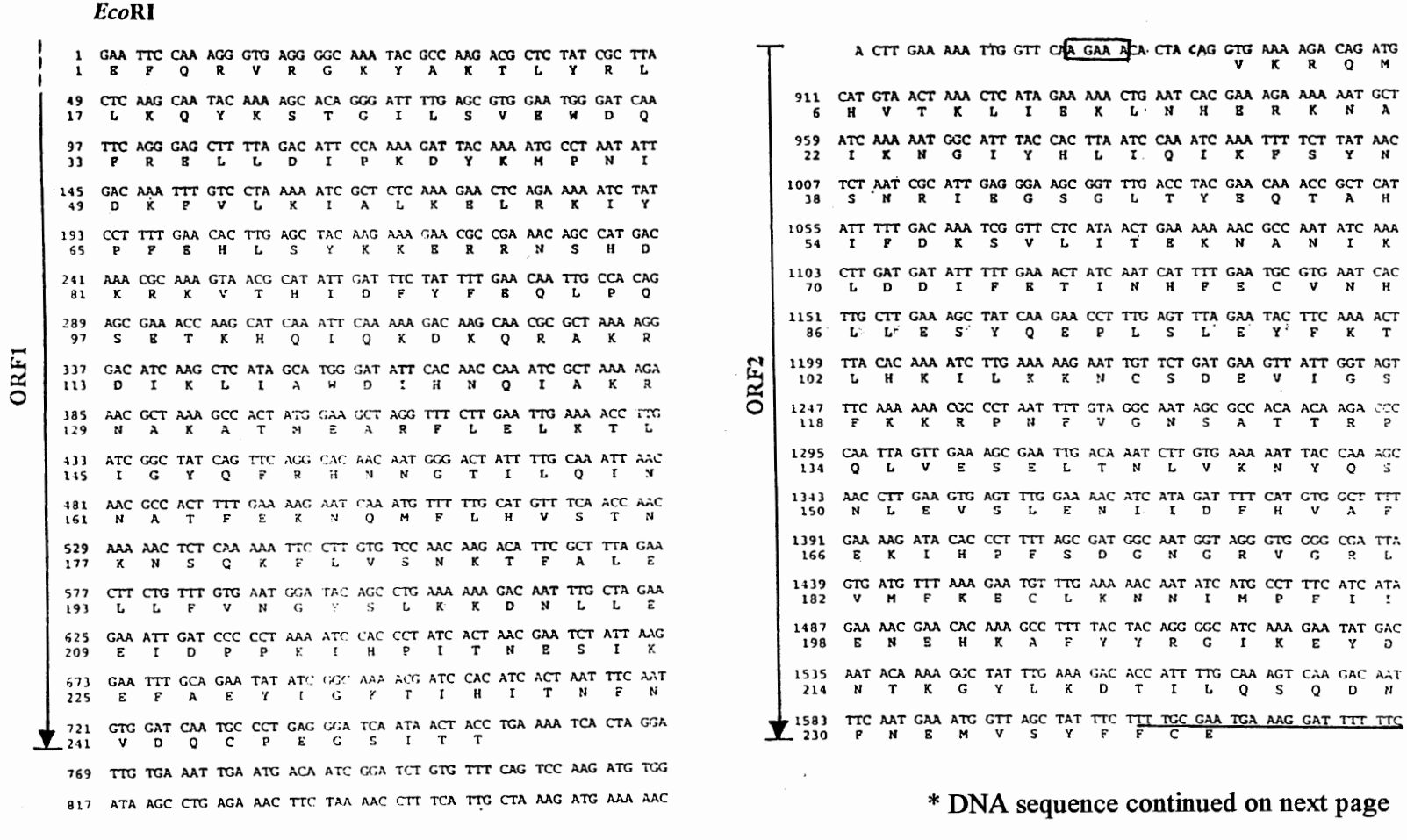


Fig. 7. Illustration of the strategy used for DNA sequence analysis. Single stranded DNA from pMJM180a and pMJM180b are represented. UNIV is the M13/pUC forward sequencing primer. P 1, P 2, ... are 17, 19, or 20mer oligonucleotide primers.

PRIMER	SEQUENCE	POSITION OF FIRST BASE OF PRIMER
UNIV	GTAAAACGACGGCCAGT	
P 1	AGAACGCCGAAACAGCC	218
P 2	GCACCGCCTATGTTGTC	3301
P 3	TCGGCTATCAGTTCAGG	434
P 4	GGTGTTATCTTTAGGGG	3078
P 5	AGGGATCAATAACTACC	737
P 6	AACCCCCATAAACAAGG	2809
P 8	CTACTCATAGTGTTCGC	2516
P 10	ACGCTTTGCTTGTGAGG	2153
P 14	TTCATTGTTAGATGCGC	1845
P 16	GACTTTGCAAAATGGTG	1574
P 17	GTTTGAAAGCGTAGGCG	2198
P 19	GCGAACACTATGAGTAG	2494
P 21	AGAAAAACTGAATCACG	944
P 22	CCCATTAGTTGAAAGCG	1310
P 23	TCAATGAAATGGTTAGC	1600
P 26	ACGCGCTACCACAAACC	2933
P 27	TCAAATCCAACCTAACC	3248
P 29	TCAGGCTGTATCCATTC	604
P 30	GATGCTTGGTTTCGCTC	304
P 34	CACAAGATTTGTCAATTCGC	1327
P 35	GGATTAAGTGGTAAATGCC	986
P 36	CCTAATCATCAGTTTGCG	1881

TABLE I. Primers utilized in the DNA sequene analysis of pHPM180. Sequences are shown 5' to 3'. Positions of the first base of each primer with respect to the double stranded sequence starting with the first base of the unique *Eco*RI site are shown in the far right column. UNIV = M13/pUC forward sequencing primer.



* DNA sequence continued on next page

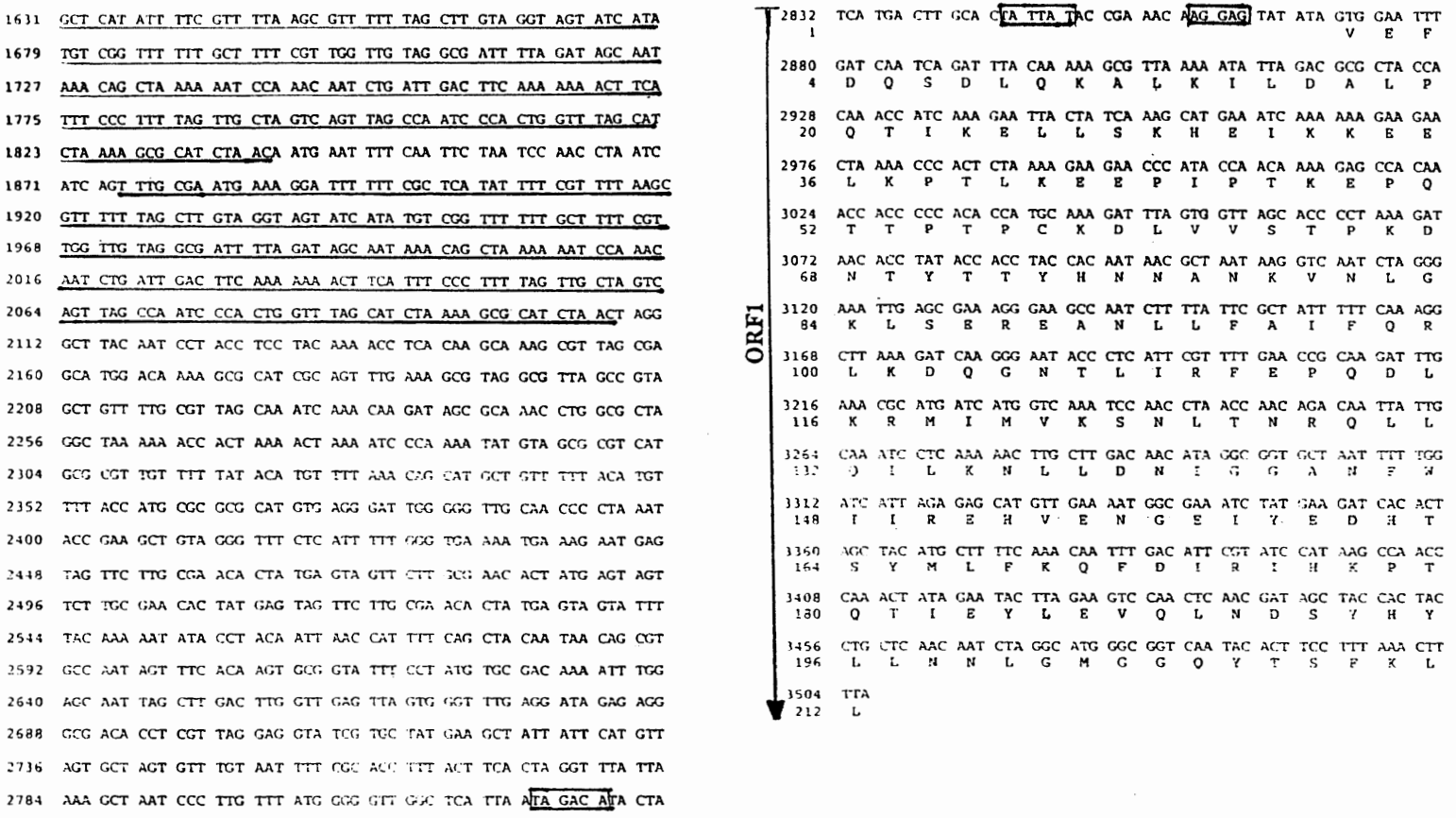


Fig. 8. DNA sequence of pHPM180. The sequence begins at the first nucleotide (nt) of the unique *Eco*RI site used for cloning and sequencing in M13mp18. A 232 base pair direct repeat is underlined. Two open reading frames (ORFs) are indicated: ORF1 from nt 2871 to nt 753; ORF2 from nt 896 (using GUG start codon) or nt 908 (using AUG start codon) to nt 1615. Translation products of ORF1 and ORF2 are indicated by single letter code. Putative ribosome binding sequences for ORF1 and ORF2, and putative promoter sequences upstream from ORF1 are boxed.

amino acids with a molecular weight of 54517. ORF2 could encode a 240 amino acid polypeptide of 28142 Da.

There was extensive DNA sequence homology between pHPM180 ORF1 and a 684 base pair *Hin*dIII fragment from another *H. pylori* plasmid, pHPM179 (Fig. 9 and see discussion). Two regions of ~180 bp were highly homologus and contained shorter homologus regions of 85, 73, 64, 44, 40, 23, and 20 bp. These bases are underlined in Fig. 9. Homology also was found between pHPM180 DNA and a 1.5 kbp *H. pylori* plasmid (pHPK255) isolated and sequenced by Kleanthous *et al.* (16) that encodes a 25 kDa replication protein (Fig. 10). Figure 11 illustrates the relative positions of ORFs, direct repeats, and sequence homology between other plasmids and pHPM180.

Identification of a transcription product from pHPM180 ORF1. To determine if ORF1 encodes transcription products in *H. pylori* HPM180, a ribonuclease protection assay (RPA) was performed. Since extensive sequence identity was found between pHPM180 ORF1 DNA and the 684 bp DNA fragment of pHPM179, the fragment from pHPM179 was considered a suitable template to make an RNA probe for ORF1 transcription products. A recombinant plasmid, pHPM376, containing the 684 bp fragment of pHPM179 cloned into the T3/T7α18 transcription vector, allowed synthesis of RNA that was complementary to ORF1 transcripts. Figure 12 illustrates the largest areas of protection (~180 base pairs) that were expected based on the DNA sequences. Autoradiography in Fig. 13 showed the expected 180 base fragments along with smaller fragments of ~ 85, 70, 60, 40, and 20 bp (tracks 11, 12, 13, and 14). Areas of identity between ORF1 and the 684 bp fragment of pHPM179, underlined in Fig. 9, could correspond to the protected fragments seen in Fig. 13. The β-actin positive control showed expected protected fragments in tracks 5 - 8. Negative control yeast RNA,

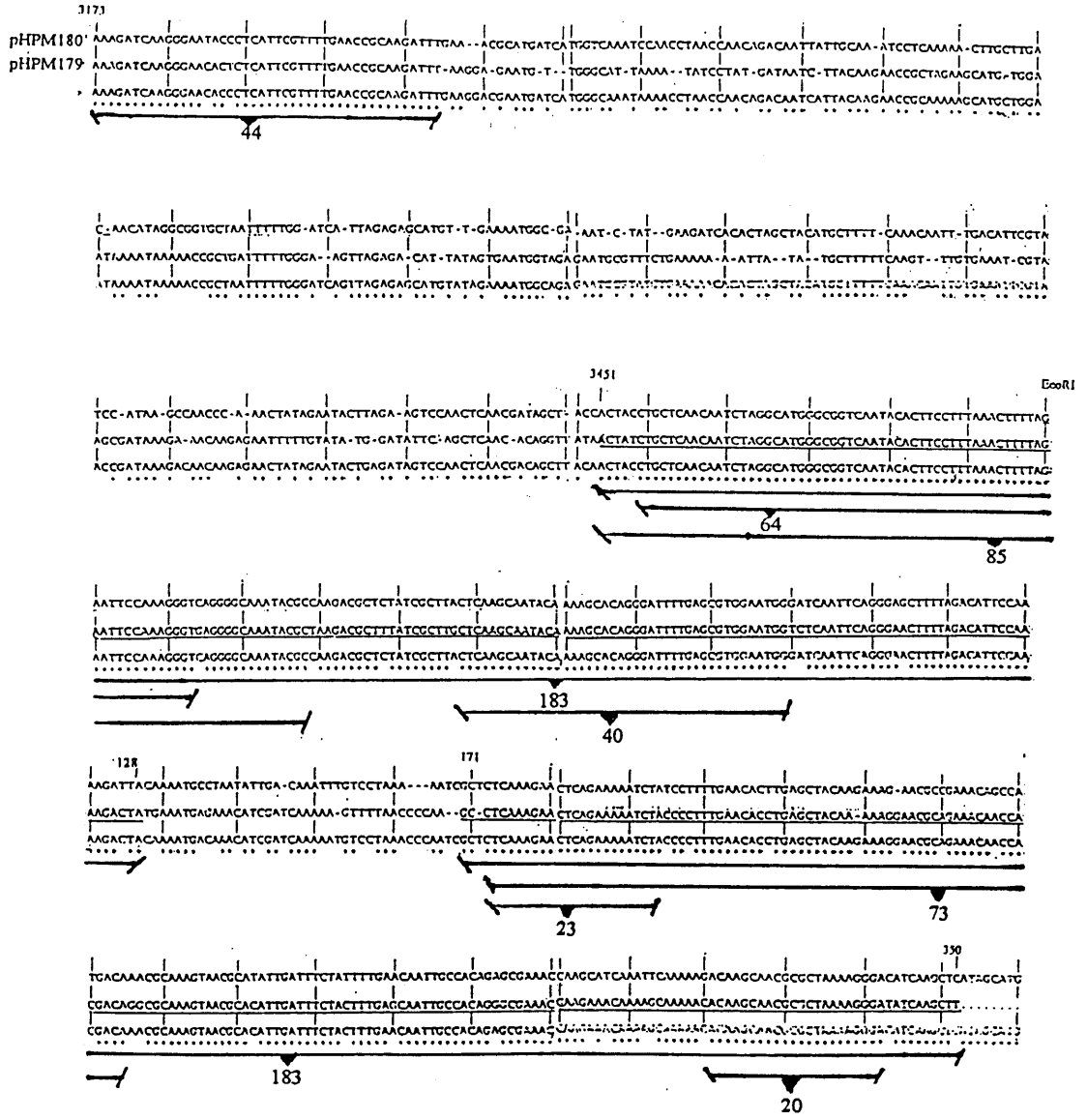


Fig. 9. Comparison of pHPM180 ORF1 and 684 base pair fragment. Alignment was performed using the DNASIS computer program. Dots show conserved nucleotides. Highly homologus regions are underlined.

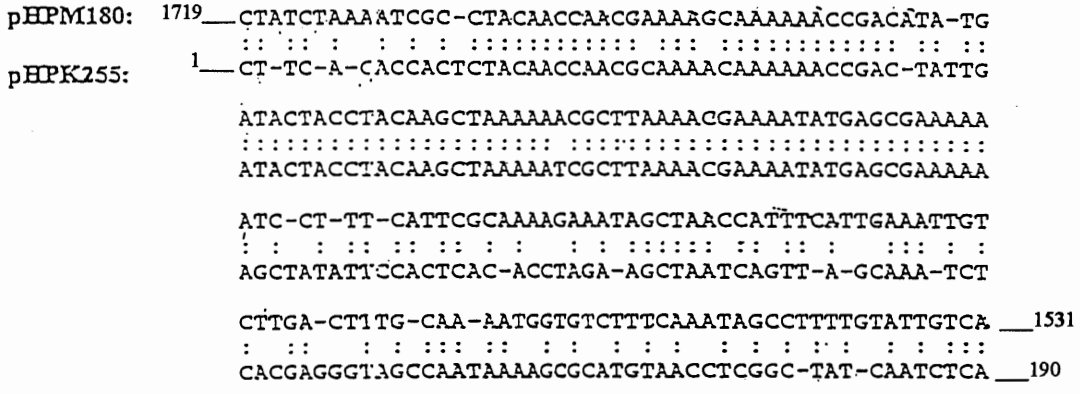


Fig. 10. Comparison of pHPM180 and pHPK255. Colons indicate the142 bases of a 200 nucleotide overlap that are identical. Nucleotide base numbers for pHPM180 correspond to the coordinates in Fig. 8; pHPK255 base numbers are from reference 16.

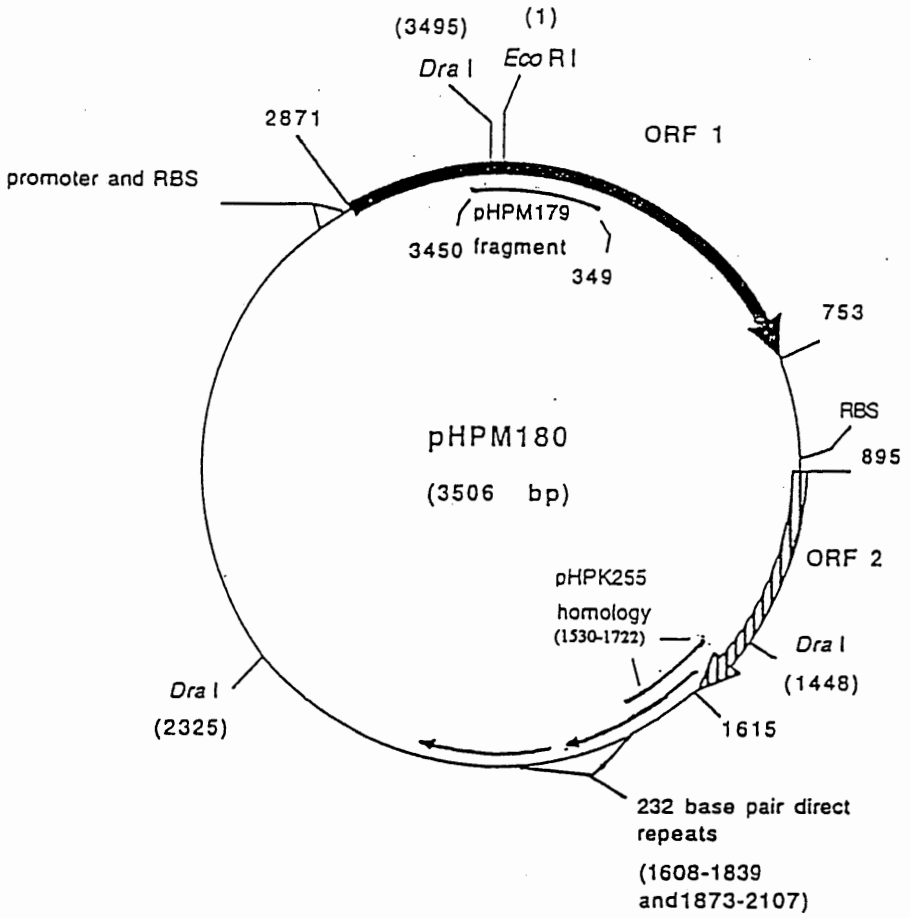


Fig. 11. Structural map of pHPM180. The relative positions of restriction enzyme cutting sites, ORFs, direct repeats, and locations of nucleotide sequence homology between pHPM180 and both the pHPM179 684 base pair fragment and the 200 bases of pHPK255 are indicated. The numbers refer to pHPM180 nucleotide positions.

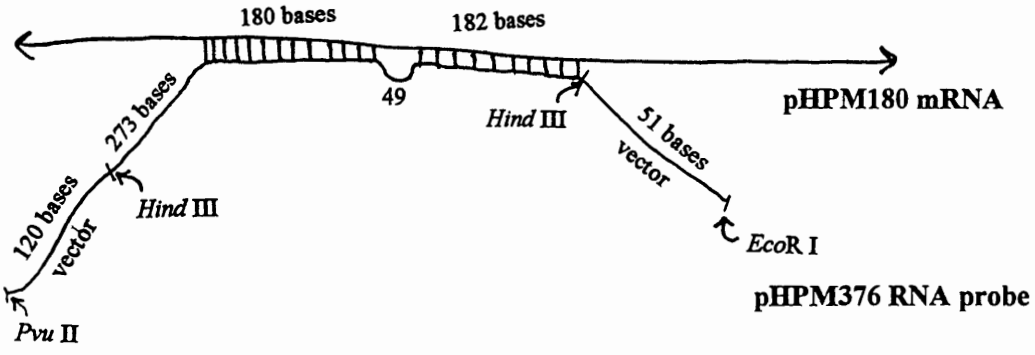


Fig. 12. Ribonuclease protection assay (RPA) hybridization diagram. The180 base regions of expected homology between pHPM180 mRNA from ORF1 and the pHPM376-derived antisense RNA probe are indicated. *Pvu*II, *Hin*dIII, and *Eco*RI represent the sites found in the corresponding DNAs.

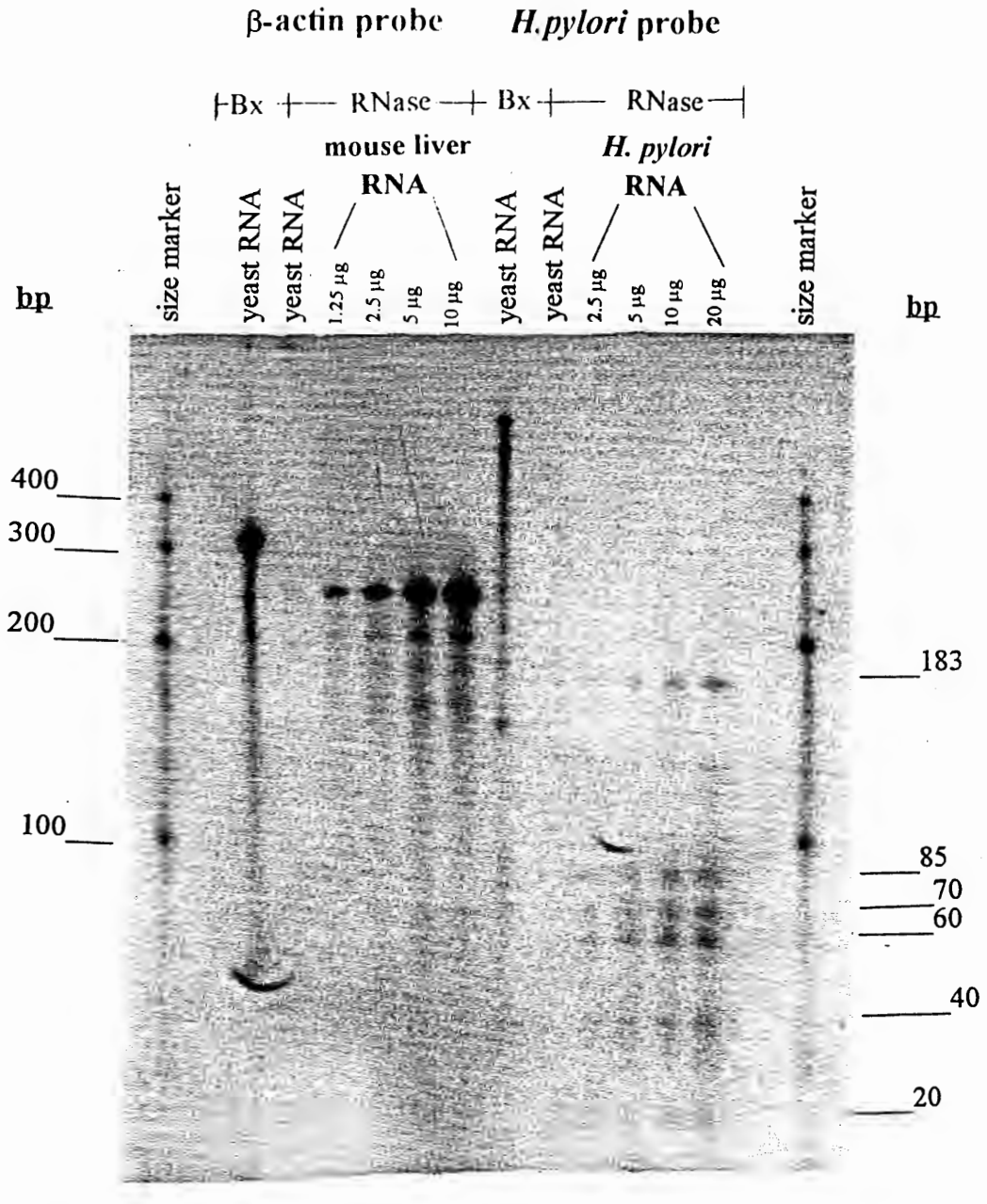


Fig. 13. Detection of an RNA transcript of pHPM180 by RPA. Autoradiogram of protected samples separated on a 0.5% (w/v) polyacrylamide/8 M urea gel. β-actin and *H. pylori* probes were labeled as described in Materials and Methods. Mouse liver RNA and yeast RNA are positive and negative controls, respectively. Bx is RNase dilution buffer. Increasing amounts of mouse liver RNA and *H. pylori* RNA were applied as indicated at the top of the gel. The size markers are indicated on the left of the figure. Estimated sizes for *H. pylori* protected fragments are indicated on the right.

hybridized with the β-actin probe and digested with RNase, showed no protected fragments (Fig. 13 tracks 4 and 10); yeast RNA hybridized with the β-actin probe and incubated with Bx without RNase showed fragments corresponding to the probe (track 3).

To determine specificity of the pHPM376-derived RNA probe with the mRNA from pHPM180 ORF1, a Southern blot analysis was performed. The same probe used in the RPA was hybridized with restriction enzyme digested and undigested samples of purified plasmid DNA (pHPM180) and total cellular DNA (chromosome plus plasmid) from HPM180. The gel picture and autoradiograph are shown in Fig. 14.

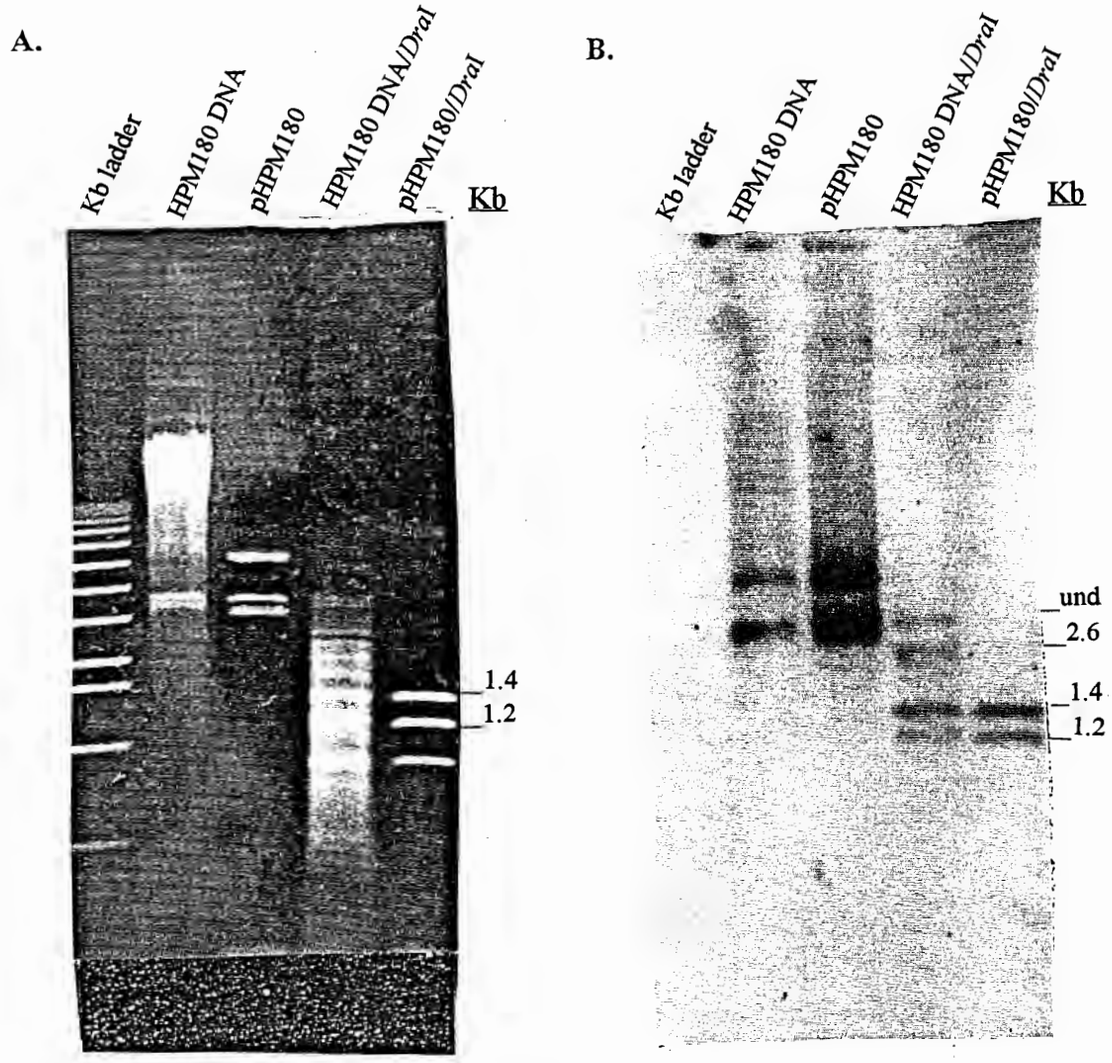


Fig. 14. A. Confirmation of RPA probe. 0.7% (w/v) agarose gel showing digested and undigested samples of purified plasmid (pHPM180) and total DNA (chromosome plus plasmid) from HPM180.

B. Autoradiogram of Southern blot of the gel in Fig. 14A showing fragments that hybridized with the pHPM376 derived antisense RNA probe used in the RPA.
Und = undigested DNA.

CHAPTER IV

DISCUSSION

Kleanthous *et al.* (16) characterized a 1.5 kbp plasmid (pHPK255) from *H. pylori*. This plasmid contained an ORF for a replication protein similar to those found in Gram-positive bacteria, but contained no other ORFs. McIntire *et al.* (22) introduced a 684 bp fragment of a 7.2 kbp *H. pylori* plasmid into *E. coli* using the pT3/T7α18 transcription vector (construct = pHPM376). The original 7.2 kbp plasmid (pHPM179) was isolated from *H. pylori* strain HPM179, obtained from a stomach antrum biopsy of a patient diagnosed with active duodenal ulcer at the V. A. Medical Center in Dallas, Tx. The DNA sequence of the 684 bp fragment of pHPM179 was determined by Chen (5), and did not contain significant DNA sequence homology with pHPK255. Computer analysis (DNASIS) of this sequence did suggest, however, that the 684 bp fragment was derived from the middle of an ORF. Further computer comparisons to known DNA and amino acid sequences of proteins in databases (Genbank and Swiss Protein) did not suggest a clear function for the deduced partial polypeptide encoded by the 684 bp fragment of pHPM179.

The present study was undertaken to gain further insight into the role of *H. pylori* plasmids. A 3.5 kbp plasmid, carried by *H. pylori* strain HPM180, obtained from a stomach fundus biopsy of a patient diagnosed with inactive duodenal ulcer at the V. A. Medical Center in Dallas, was chosen for characterization. This plasmid was larger than the previously characterized pHPK255 plasmid from *H. pylori*, and was, therefore, thought to afford a greater potential for discovery of new ORFs and significant DNA

sequence information.

The pHPM180 DNA was introduced into *E. coli* DH5αF' using the M13mp18 vector. Previous attempts to clone *H. pylori* plasmid DNA had produced only a few recombinant clones. It is possible that *H. pylori* plasmid DNA contains modifications, such as unusual methylation patterns, that are recognized by *E. coli* host strains as foreign DNA, and the incoming DNA may be rapidly degraded by host enzymes. Since the double-stranded DNA from phage M13 derivatives rapidly forms restriction resistant single-stranded DNA for replication and packaging in the host cells, cloning into M13-derived vectors was considered as a possible solution to the restriction problem (J. Knesek, pers. comm.). Use of the vector M13mp18 generated the desired clone (14s) carrying *H. pylori* DNA at a frequency of approximately 0.05.

Digestion and religation of recombinant 14s successfully generated two clones (pMJM180a and pMJM180b) thought to contain pHPM180 DNA in opposite orientations. Labeled probes were derived from pMJM clones (see Methods) and hybridized with digested clone, vector, and pHPM180 DNAs (Fig. 6). This hybridization confirmed that pMJM180a and pMJM180b contained pHPM180 DNA. Having pHPM180 DNA ligated into M13mp18 in both orientations allowed isolation of single-stranded DNA from each clone for DNA sequence determination. Each single-stranded clone contained a different strand of pHPM180 DNA. Thus, DNA sequence analysis of both clones produced sequence for both strands of pHPM180 DNA. Analysis was performed by initially utilizing the universal M13/pUC forward sequencing primer followed by subsequent primers designed from each previously determined sequence information. Sequence data were entered into DNASIS for analysis, and the DNA

connecting program was used to test each proposed new primer for other possible priming sites. During primer analysis, one proposed primer was observed to prime two areas of pHPM180. This led to the discovery of a 232 bp direct repeat (discussed below) in the pHPM180 DNA sequence. Compilation of the sequencing data revealed that pHPM180 was composed of 3506 nucleotide base pairs, which makes it the largest plasmid sequenced in *H. pylori*. Furthermore, pHPM180 is the only other entire plasmid from *H. pylori*, besides pHPK255, to be cloned and the DNA sequence analyzed.

Analysis of the complete nucleotide sequence of pHPM180 (Fig. 8) revealed two ORFs. ORF1, beginning at the GUG codon at nucleotide 2871, could be translated to 463 amino acids producing a protein of molecular weight 54,517. Upstream from the ORF1 translation start codon (GUG), were a ribosome binding consensus sequence (AGGAG at nucleotide 2860), and -10 and -35 promoter consensus sequences at nucleotides 2855 and 2821 (TATTAT and TAGACA, respectively). ORF2, beginning at a GUG translation initiation codon at nucleotide 896, could be translated to 240 amino acids producing a protein of molecular weight 28,142. A ribosome binding consensus sequence of AGAAA is located at nucleotide 883. Translation of ORF2 could also be initiated at an AUG codon at nucleotide 908. Lack of promoter consensus sequences upstream of ORF2 could indicate that a single polycistronic mRNA is produced from the ORF1 promoter. The promoter and ribosome binding consensus sequences that were found in the pHPM180 sequence corresponded to the *E. coli* ribosome binding consensus sequence of AGGAG and the -10 and -35 promoter consensus sequences of TATTAT and TTGACA, respectively.

Nucleotide sequence analysis of pHPM180 DNA also revealed a 232 bp direct repeat interrupted by 35 bases of nonrepeating DNA (Fig. 8). This repeat includes the

last eight nucleotides of ORF2 DNA and extends into the approximately 1200 nucleotides of pHPM180 that are presumably involved in plasmid replication and maintenance. At present the function of this repeat is unknown. Since restriction patterns of *H. pylori* chromosomal and plasmid DNAs are highly variable between individuals (27), there may be significant DNA rearrangements occuring with *H. pylori* DNA. The repeat may have resulted from such DNA rearrangements, although repeated subcloning of plasmid-containing strains over 3-4 years does not produce changes in DNA patterns. Therefore, the source of variability among *H. pylori* strains remains unknown.

Greater than 80% homology was found between the DNA sequences of pHPM180 ORF1 and the 684 bp fragment of pHPM179 (Fig. 9). Analysis of the 684 bp fragment with DNASIS indicated that it was part of an ORF. In addition, searches of the data bases for amino acid and DNA sequence homologies to known sequences revealed 71% homology (Fig. 10) between a 200 nucleotide region of pHPM180 and the 1.5 kbp plasmid, pHPK255, of Kleanthous *et al.* (15). This region of homology was not contained within the ORF of pHPK255 but contained the last 85 nucleotides of pHPM180 ORF2 and the first portion of the 232 bp direct repeat (Fig. 11). Conservation of these two regions could reflect their essential roles in *H. pylori* plasmid biology. Proteins encoded by ORF1 of pHPM180 and the ORF encompassed by the 684 bp fragment of pHPM179 may be important for replication or maintenance of the plasmids, or alternatively, could be involved in pathogenisis. The conserved regions of pHPM180 and pHPK255 may be non-coding areas important in replication or maintenance of the plasmids. Completion of the pHPM179 sequence and DNA analysis of additional *H. pylori* plasmids may provide the information needed to determine the function(s) of these conserved regions.

As a first step toward elucidating the function of ORF1, a ribonuclease protection assay (RPA) was used to determine whether ORF1 was transcribed in *H. pylori* strain HPM180 (Fig. 13). Availability of plasmid pHPM376 carrying the 684 bp fragment of pHPM179 ligated in a T3/T7α18 transcription vector provided a means of generating a probe that would have significant and definable identity to mRNA transcribed from ORF1. Analysis of nucleotide sequences of both DNAs indicated that two regions of approximately 180 nucleotides were nearly identical and should be protected in the RPA (see Fig. 12). In addition, smaller regions of identity or near identity could be protected in the RPA (see underlined sequences of Fig. 9). Therefore, an antisense RNA probe was generated from the pHPM376 construct and was hybridized with total RNA isolated from HPM180 cells. The autoradiogram depicted in Fig. 13 clearly showed the expected protected fragments of ~183, 85, 70, 60, 40, and 20 bp of *H. pylori* RNA hybridized with the probe. These hybridization results also showed that the protected bands increased in intensity with increasing amounts of total RNA hybridized with the probe (which was in molar excess). The positive β-actin controls and negative yeast RNA controls were as expected in the assay.

An additional control experiment (shown in Fig. 14) confirmed that the antisense RNA probe derived from HPM376 hybridized with the expected fragments of pHPM180 DNA and did not hybridize with *H. pylori* chromosomal DNA. The *Dra*I digestion of plasmid DNA in track 5 of both A and B showed that fragments of 1.4 and 1.2 kbp hybridized with the probe as expected. In the total DNA *Dra*I digest (track 4), some undigested plasmid DNA and a fragment of 2.6 kbp hybridized with the probe. Since no chromosomal DNA hybridized in track 2, the 2.6 kbp fragment was presumed to be a partial fragment composed of the 1.4 and 1.2 kbp fragments of the plasmid. The

conclusion was that the antisense probe derived from the 684 bp fragment of pHPM179 was specific for the plasmid DNA of pHPM180 and did not hybridize with any chromosomal DNA.

With the DNA sequence of pHPM180 available, new avenues of research on *H. pylori* plasmids can now be explored. Even though the replication region was not clearly identified, this plasmid can be used to generate a shuttle vector which could transport DNA of interest into different strains of *H. pylori*. Further characterization of the polypeptides encoded by ORF1 and ORF2 may reveal useful information about novel proteins encoded by the *H. pylori* plasmids. These proteins may help to identify the function of these plasmids and further aid in understanding *H. pylori* pathogenicity.

REFERENCES

1. Armstrong, J. A., S. H. Wee, C. S. Goodwin, and D. H. Wilson. 1987. Response of *Campylobacter pyloridis* to antibiotics, bismuth and an acid-reducing agent in vitro ---an ultrastructural study. J. Med. Microbiol. 24:343-350.
2. Birnboim, H., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513-1523.
3. Blaser, M. J. 1990. *Helicobacter pylori* and the pathogenesis of gastroduodenal inflammation. J. Infect. Dis. 161:626-633.
4. Cave, D. R., and M. Vargas. 1989. Effect of a *Campylobacter pylori* protein on acid secretion by parietal cells. The Lancet 2:187-189.
5. Chen, J. 1993. M. S. Thesis. Texas Woman's University, Denton, Tx.
6. Chittajallu, R. S., C. A. Dorrian, J. E. S. Ardill, and K. E. L. McColl. 1992. Effect of *Helicobacter pylori* on serum pepsinogen I and plasma gastrin in duodenal ulcer patients. Scand. J. Gastroenterol. 27:20-24.
7. Cover, T., W. Puryear, G. Perez-Perez and M. Blaser. 1991. Effect of urease on HeLa cell vacuolation induced by *Helicobacter pylori* cytotoxin. Infect. Immun. 59:1264-1270.
8. Drumm, B., G. I. Perez-Perez, M. J. Blaser, and P. M. Sherman. 1990. Intrafamilial clustering of *Helicobacter pylori* infection. N. Eng. J. Med. 322:359-363.
9. Dunn, B. E., G. P. Campbell, G. I. Perez-Perez, and M. J. Blaser. 1989. Purification and characterization of urease from *Helicobacter pylori*. J. Biol. Chem. 265:9464-9469.
10. Dunn, B. E., R. M. Roop II, C. Sung, S. A. Sharma, G. I. Perez-Perez, and M. J. Blaser. 1992. Identification and purification of a cpn60 heat shock protein homolog from *Helicobacter pylori*. Infect. Immun. 60:1946-1951.
11. Dworkin, B. M., J. E. Chodos, M. E. Fernandez, K. Van Horn, F. Cabello, and G. P. Wormser. 1991. Use of plasmid profiles in the investigation of a patient with *Helicobacter pylori* infection and peptic ulcer disease. Amer. J. Gastroenterol. 86: 354-356.
12. Goodwin, C., J. Armstrong, T. Chilvers, M. Peters, M. Collins, L. Sly, W. McConnell, and W. Harper. 1989. Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. Int. J. Syst. Bacteriol. 39: 397-405.

13. Gregson, D. B., D. E. Low, M. M. Cohen, N. B. Cooter, J. J. Connon, S. L. Wolman, and A. E. Simor. 1989. The prevalence of *Campylobacter pylori* gastritis among asymptomatic adults. CMAJ 140:1449-1453.
14. Hazell, S. L., W. B. Hennesy, T. J. Borody, J. Carrick, M. Ralston, L. Brady, and A. Lee. 1987. *Campylobacter pyloridis* gastritis II: distribution of bacteria and associated inflammation in the gastroduodenal environment. Amer. J. Gastroenterol. 82:297-301.
15. Hazell, S., A. Lee, L. Brady and W. Hennessy. 1986. *Campylobacter pyloridis* and gastritis: association with intracellular spaces and adaptation to an environment of mucus as important factors in colonization of the gastric epithelium. J. Infect. Dis. 153:658-663.
16. Kleanthous, H., C. L. Clayton, and S. Tabaqchali. 1991. Characterization of a plasmid from *Helicobacter pylori* encoding a replication protein common to plasmids in Gram-positive bacteria. Molec. Microbiol. 5:2377-2389.
17. Lee, A., J. Fox, and S. Hazell. 1993. Pathogenicity of *Helicobacter pylori:* a perspective. Infect. Immun. 61:1601-1610.
18. Levi, S., K. Beardshall, G. Haddad, R. Flaford, P. Ghosh and J. Calam. 1989. *Campylobacter pylori* and duodenal ulcers: the gastrin link. Lancet i:1167-1168.
19. Malaty, H. M., D. Y. Graham, P. D. Klein, D. G. Evans, E. Adams, and D. J. Evans. 1 991. Transmission of *Helicobacter pylori* infection. Scand. J. Gastroenterol. 26:927 -932.
20. Marshall, B., L. Barret, C. Prakesh, R. McCallum and R. Guerrant. 1988. Protection of *Campylobacter pyloridis* but not *Campylobacter jejuni* against acid susceptibility by urea, p. 402-403. In B. Kaijser and E. Falsen (ed), Campylobacter IV. University of Goteborg, Goteborg, Sweden.
21. Marshall, B. and J. Warren. 1983. Spiral bacteria in the human stomach: a common finding in patients with gastritis and duodenal ulcer. pp. 33-38. *In Campylobacter* II. Pearson, A. D., Skirrow, M. B., Rove, B., Davies, J., and Jones, D. M. (eds). Proceedings of the Second International Workshop on *Campylobacter* Infections, Public Health Laboratory Service, London.
22. McIntire, S. A., J. Knesek, and S. Montanez. 1991. Plasmids of *Helicobacter pylori*. Prog. Abstr., p. 182, 91st Gen. Mtg. of the Am. Soc. Microbiol., Dallas, Tx., May, 1991.
23. McIntire, S. A., and W. L. Peterson. 1990. Analysis of plasmid DNA in clinical isolates of *Helicobacter pylori*. Prog. Abstr., p. 36, The Ann. Mtg. of the Am. Gastroenterol. Assoc., San Antonio, Tx., May, 1990.
24. Penfold, S., A. Lastovica and B. Elisha. 1988. Demonstration of plasmids in *Campylobacter pylori.* J. Infect. Dis. 157: 850-851.
25. Slomiany, B., J. Bilski and J. Sarosiek. 1987. *Campylobacter pylori* degrades mucin and undermines gastric mucosal integrity. Biochem. Biophys. Res. Commun. 144: 307-314.

26. Talley, N. J., A. R. Zinsmeister, A. Weaver, E. P. DiMagno, H. A. Carpenter, G. I. Perez-Perez, and M. J. Blaser. 1991. Gastric adenocarcinoma and *Helicobacter pylori* infection. J. Natl. Cancer Inst. 83:1734-1739.
27. Taylor, D. N. 1992. Genetics of *Campylobacter* and *Helicobacter*. Annu. Rev. Microbiol. 46:35-64.
28. Taylor, D. N., and M. J. Blaser. 1991. The epidemiology of *Helicobacter pylori* infection. Epidemiol. Rev. 13:42-59.
29. Tjia, T. N., W. E. S. Harper, C. S. Goodwin, W. B. Grubb. 1987. Plasmids in *Campylobacter pyloridis*. Microbios. Lett. 36:7-11.
30. Tummuru, M. K. R., T. L. Cover, and M. J. Blaser. 1993. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. Infect. and Immun. 61:1799-1809.
31. Warren, J. R. 1983. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet i: 1273.