

## Cloning and Sequence Analysis of Two Copies of a 23S rRNA Gene from *Helicobacter pylori* and Association of Clarithromycin Resistance with 23S rRNA Mutations

DIANE E. TAYLOR,<sup>1,2\*</sup> ZHONGMING GE,<sup>1</sup> DALE PURYCH,<sup>1</sup> TONY LO,<sup>1</sup> AND KOJI HIRATSUKA<sup>1,2</sup>

Departments of Medical Microbiology and Immunology<sup>1</sup> and Biological Sciences,<sup>2</sup>  
University of Alberta, Edmonton, Alberta, Canada

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In this study, two identical copies of a 23S-5S gene cluster, which are separately situated within the *Helicobacter pylori* UA802 chromosome, were cloned and sequenced. Comparison of the DNA sequence of the *H. pylori* 23S rRNA gene with known sequences of other bacterial 23S rRNA genes indicated that the *H. pylori* UA802 23S rRNA genes are closely related to those of *Campylobacter* spp. and therefore belong in the proposed *Proteobacteria* subdivision. The 5'-terminal nucleotide T or A of the 23S rRNA is close to a Pribnow box which could be a -10 region of the transcription promoter for the 23S rRNA gene, suggesting that a posttranscriptional process is likely not involved in the maturation of the *H. pylori* 23S rRNA. Clinical isolates of *H. pylori* resistant to clarithromycin were examined by using natural transformation and pulsed-field gel electrophoresis. Cross-resistance to clarithromycin and erythromycin, which was transferred by natural transformation from the *Cla*<sup>r</sup> *Ery*<sup>r</sup> donor strain *H. pylori* E to the *Cla*<sup>s</sup> *Ery*<sup>s</sup> recipient strain *H. pylori* UA802, was associated with a single A-to-G transition mutation at position 2142 of both copies of the 23S rRNA in UA802 *Cla*<sup>r</sup> *Ery*<sup>r</sup> mutants. The transformation frequency for *Cla*<sup>r</sup> and *Ery*<sup>r</sup> was found to be approximately  $2 \times 10^{-6}$  transformants per viable cell, and the MICs of both clarithromycin and erythromycin for the *Cla*<sup>r</sup> *Ery*<sup>r</sup> mutants were equal to those for the donor isolate. Our results confirmed the previous findings that mutations at positions 2142 and 2143 of the *H. pylori* 23S rRNA gene are responsible for clarithromycin resistance and suggest that acquisition of clarithromycin resistance in *H. pylori* could also result from horizontal transfer.

*Helicobacter pylori* is accepted as the cause of chronic gastritis and plays a role in the development of both duodenal and gastric ulcers (2, 9) as well as gastric carcinoma (26, 29). Although *H. pylori* is susceptible to most antimicrobial agents in vitro, in vivo eradication of this pathogen has been difficult (18). The highest cure rates have required multidrug antimicrobial therapies including combinations of clarithromycin, metronidazole, or amoxicillin in association with a proton pump inhibitor, e.g., omeprazole (32, 43).

The prevalence of *H. pylori* resistant to clarithromycin varies with geographic location. In Alberta, Canada, fewer than 1% of *H. pylori* strains are resistant (41). In France and Belgium, two countries where the use of macrolide antibiotics is known to be higher, the percentage of *H. pylori* strains resistant to clarithromycin has been reported to be 10% (18). The prevalence of clarithromycin-resistant *H. pylori* in patients in whom treatment with this particular antibiotic failed was reported to be between 20 and 60% (4).

Previous studies have examined clarithromycin-resistant *H. pylori* isolates (7, 44). Sequencing of PCR products prepared with primers complementary to the 23S rRNA genes from *Escherichia coli* (44) or *H. pylori* (7) demonstrated mutations at two positions in the domain V loop of 23S rRNA: adenosine at position 2058 or 2059 to guanosine, using the *E. coli* coordinates (27, 35, 36, 44, 45), and A2514 or A2515 to G, using *H. pylori* coordinates (7). Recently, Stone et al. (35, 37) reported that resistance to clarithromycin in *H. pylori* can also be con-

ferred by an A-to-C transversion at position 2143 of the 23S rRNA.

The goal of this study was to determine the DNA sequences of the two copies of the 23S rRNA gene from *H. pylori* and compare 23S rRNA sequences from clarithromycin-resistant strains. We also used natural transformation to transfer clarithromycin and erythromycin resistance determinants to susceptible strains and examined the DNA sequence variation of the 23S rRNA genes using pulsed-field gel electrophoretic analysis and DNA sequencing.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The *H. pylori* strains used in this study are listed in Table 1 and were grown on BHI-YE agar (3.7% brain heart infusion [BHI] agar base with 0.3% yeast extract and 5% animal serum) under microaerobic conditions (5% CO<sub>2</sub>, 5% H<sub>2</sub>, 90% N<sub>2</sub>) as described previously (15). The vectors  $\lambda$ -DASH II and Bluescript II SK<sup>+</sup> (Stratagene, La Jolla, Calif.) and *E. coli* SRB (Stratagene) and DH10B (Gibco/BRL Life Technologies, Gaithersburg, Md.) were used in the construction of a genomic DNA library and subcloning of the genes of interest, respectively. Luria-Bertani (LB) medium (broth or agar) (33) was used to grow *E. coli* cells. The solid and liquid LB media were supplemented with ampicillin (100  $\mu$ g/ml) for growing plasmid-containing cells.

**DNA manipulation techniques.** Chromosomal DNA from *H. pylori* was isolated by the method of Ezaki et al. (12). Plasmid DNA was prepared by the procedure developed by Birnboim and Doly (1). DNA sequencing was carried out by using the thermocycling sequencing system with Thermo-Sequenase, purchased from Amersham Life Sciences, Cleveland, Ohio. Southern blotting and hybridization were performed as described previously (33). Probes for DNA hybridization were labeled with [<sup>32</sup>P]dCTP (DuPont Canada Inc., Mississauga, Ontario, Canada) by using the random primer labeling system (Gibco/BRL).

**Cloning of the 23S rRNA genes from *H. pylori*.** A genomic DNA library of *H. pylori* UA802 was constructed by using the  $\lambda$ -DASH II vector as detailed previously (21). Two clones, clones  $\lambda$ 23.54 and  $\lambda$ 23.B1, were demonstrated to contain the *H. pylori* 23S rRNA genes by in situ hybridization with a radioactively labeled probe. For the preparation of the probe, two primers, primers 23.5b (5'-GTCCGGTAAGTCCGACCTG-3') and 23.3b (5'-GGCGAACAGCCATACCCCTG-3'), were generated from the nucleotide sequence of the *E. coli* 23S rRNA gene (11) and were used for PCR amplification of a corresponding region of the

\* Corresponding author. Mailing address: Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. Phone: (403) 492-4777. Fax: (403) 492-7521. E-mail: diane.taylor@ualberta.ca.

TABLE 1. MICs of clarithromycin resistant strains used in this study

| Strain                    | Origin                    | MIC ( $\mu\text{g/ml}$ ) |                | Susceptibility <sup>a</sup> |
|---------------------------|---------------------------|--------------------------|----------------|-----------------------------|
|                           |                           | Erythromycin             | Clarithromycin |                             |
| A                         | Dublin, Ireland           | 256–512                  | 4–8            | R                           |
| B                         | Dublin, Ireland           | 64–128                   | 0.5–1          | I                           |
| D                         | Dublin, Ireland           | 256–512                  | 8–16           | R                           |
| E                         | Dublin, Ireland           | 256–512                  | 16–32          | R                           |
| MQ                        | Victoria, Australia       | 256–512                  | 16–32          | R                           |
| UA802                     | Edmonton, Alberta, Canada | 0.06–0.25                | 0.002–0.008    | S                           |
| UA802 Ery <sup>r</sup> -1 | Current study             | 512                      | 16–32          | R                           |
| UA802 Ery <sup>r</sup> -2 | Current study             | 512                      | 16–32          | R                           |
| UA802 Ery <sup>r</sup> -4 | Current study             | 512                      | 16–32          | R                           |
| UA802 Cla <sup>r</sup> -1 | Current study             | 512                      | 32             | R                           |
| UA802 Cla <sup>r</sup> -2 | Current study             | 512                      | 32             | R                           |

<sup>a</sup> R, resistant; I, intermediate; S, susceptible.

*H. pylori* 23S rRNA genes (21). A single product of 650 bp was obtained, and this product was then used to prepare a radioactively labeled probe for screening other 23S rRNA gene clones. Subsequently, an *Xba*I fragment of approximately 10 kb containing the 23S rRNA genes from the  $\lambda$  clones described above was subcloned into the vector Bluescript II SK<sup>+</sup>. Two plasmid clones, clone p54X1.1 derived from  $\lambda$ 23.45 and clone pB1X1.1 derived from  $\lambda$ 23.B1, were then sequenced.

**Primer extension.** The nucleotide sequence of the primer (the PEP primer) was complementary to the region spanning nucleotides 516 to 533 (see Fig. 2). *H. pylori* RNA was isolated by the method of Reddy and Gilman (30). The resulting RNA sample was treated with RNase-free DNase (Boehringer Mannheim/Canada, Laval, Quebec, Canada) in a DNase digestion buffer (20 mM Tris-HCl [pH 8.0], 10 mM MgCl<sub>2</sub>) at 37°C for 30 min. The DNase was then removed by passing the digestion mixture through a Chroma-spin column according to the supplier's instructions (Clontech, Mississauga, Ontario, Canada). Afterward, the RNA was precipitated with ethanol, air dried, and dissolved in 100  $\mu$ l of diethyl pyrocarbonate-treated double-distilled H<sub>2</sub>O. Primer extension was carried out as described previously (16). Five microliters of sequencing loading dye was added to the reaction mixture, and 2  $\mu$ l of the samples along with the corresponding sequencing reactions prepared by using p54X1.1 DNA and the PEP primer were separated on a 5% polyacrylamide sequencing gel.

**MIC testing and natural transformation.** The MICs for the *H. pylori* strains were determined on BHI-YE agar plates containing different concentrations of clarithromycin and erythromycin. Introduction of the clarithromycin-resistant determinants into a susceptible strain by natural transformation was performed on a BHI-YE agar surface as described previously (46). Briefly, recipient cells were heavily inoculated onto cold BHI-YE agar plates and were grown for 4 to 10 h; this was followed by the addition of 0.5 to 1  $\mu$ g of donor DNA to the bacterial lawn; subsequently, the cells grew for 1 to 2 days and Cla<sup>r</sup> transformants were selected on BHI-YE agar plates containing either erythromycin (32  $\mu\text{g/ml}$ ) or clarithromycin (2  $\mu\text{g/ml}$ ). To determine the frequency of such transformation, the transformed cells were removed from the agar surface, diluted in BHI broth, and plated onto BHI-YE agar containing erythromycin (16  $\mu\text{g/ml}$ ) for selection of erythromycin-resistant transformants, whereas total colony numbers were determined on regular BHI-YE agar.

**Characterization of clarithromycin-resistant *H. pylori* mutants.** The complete *H. pylori* chromosomal DNA was digested with *Not*I, and the fragments were then separated by pulsed-field gel electrophoresis as described previously (39, 40). DNA fragments containing each of two *H. pylori* rRNA gene copies were localized by hybridization of the restriction enzyme-digested DNA with the 23S rRNA gene-specific probe and were isolated from the agarose gel by the methods of Sambrook et al. (33). Two primers, primers DP1 (5'-ACGGCGGCCGTAACTATA-3' corresponding to positions 2357 to 2374) and ZGE23 (5'-ACAGGCCAGTTAGCTA-3' complementary to positions 2649 to 2664), were derived from the 23S rRNA gene, respectively (Fig. 2). The fragment of interest was amplified by PCR with the primers DP1 and ZGE23 and the DNA sequence determined from both strands.

**Analysis of the nucleotide sequence.** The nucleotide sequence reported in this study was analyzed by using the software package of GCG, version 8 (Genetics Computer Group, Madison, Wis.).

**Nucleotide sequence accession numbers.** The nucleotide sequence reported in this study has been deposited in GenBank under accession no. U27270.

## RESULTS

**Strategies for cloning two copies of the *H. pylori* 23S rRNA gene.** The  $\lambda$ -DASH-II genomic library of *H. pylori* DNA was

screened with the probe derived from a 650-nucleotide PCR DNA fragment of the 23S rRNA gene. Approximately 2,000 plaques were screened from the library, and ~7.5% of the clones hybridized with the probe. Subsequently, a clone, clone  $\lambda$ 23.54, was partially digested with *Xba*I and its *Xba*I restriction map was constructed (data not shown). The 23S rRNA gene is located on the 10-kb *Xba*I fragment of  $\lambda$ 23.54, demonstrated by hybridization with the PCR probe derived from the 23S rRNA gene (data not shown).

We previously confirmed that the *H. pylori* genome carries two copies of the 23S rRNA gene (22). Therefore, we decided to clone each individual copy of the 23S rRNA gene. A 3.0-kb *Eco*RI-*Xba*I fragment, located upstream from the 5' end of the 23S rRNA gene-containing *Xba*I fragment in the  $\lambda$ 23.54 clone, did not hybridize with the 23S probe. A probe prepared from this DNA fragment was then used for Southern hybridization with the *Xba*I-digested DNA samples from nine of the 23S rRNA gene-containing  $\lambda$ -DASH-II clones. The clones which did not hybridize with the probe could have originated from the other 23S rRNA gene region on the *H. pylori* UA802 genome. Five of these nine clones had no homology with this probe, indicating that they may have chromosomal origins different from that of  $\lambda$ 23.54. A clone, clone  $\lambda$ 23.B1, was selected for further characterization.

The 23S rRNA genes of both  $\lambda$ 23.54 and  $\lambda$ 23.B1 were located in the *Xba*I-fragments of ~10 kb (data not shown). These fragments were cloned into the pBluescript SK<sup>+</sup> at the *Xba*I site to give the plasmids p54X1.1 (derived from  $\lambda$ 23.54) and pB1X1.1 (derived from clone  $\lambda$ 23.B1). Restriction digestion of these two clones in combination with Southern hybridization with the PCR probe derived from the 23S rRNA gene indicated that the enzymes *Kpn*I, *Sst*II, and *Nru*I were located within the 23S rRNA genes of both p54X1.1 and pB1X1.1 (Fig. 1). In addition, these results also indicated that p54X1.1 and pB1X1.1 should represent different 23S rRNA gene origins, since the restriction fragment lengths of the enzymes listed above relative to those of the *Xba*I sites at both ends of the inserts in p54X1.1 and pB1X1.1 are different. The 1-kb *Sst*II-*Sst*II and the 2.5-kb *Sst*II-*Xba*I fragments from both p54X1.1 and pB1X1.1 were subcloned into the pBluescript II SK<sup>+</sup> to generate plasmids which were convenient for sequencing.

**Nucleotide sequences of two copies of the 23S rRNA gene.** DNA sequences were obtained from all of the plasmids described above by using cycle sequencing and either the T7 or T3 sequencing primer. Nested deletions of the recombinant plasmids containing *Sst*II-*Sst*II and *Sst*II-*Xba*I 23S fragments

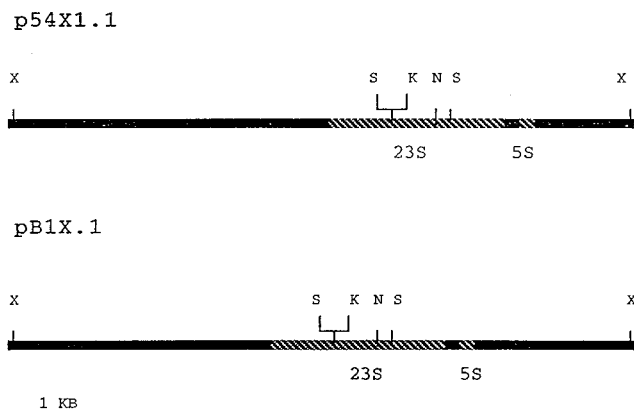


FIG. 1. Restriction maps of p54X1.1 and pB1X1.1 subcloned from  $\lambda$ 23.54 and  $\lambda$ 23.B1, respectively. The 23S and 5S rRNA genes are indicated by hatched squares. Restriction enzymes: X, *Xba*I; K, *Kpn*I; N, *Nru*I; S, *Sst*II.

were made for sequencing. Sequencing primers were synthesized when necessary. Both p54X1.1 and pB1X1.1 contain an rRNA gene cluster consisting of one copy of each of the 23S and 5S rRNA genes. The nucleotide sequences, including the 23S-5S rRNA gene as well as 372 bp flanking the 5' end of the 23S rRNA gene and 62 bp flanking the 3' end of the 5S rRNA gene, are identical in clones p54X1.1 and pB1X1.1 and are presented in Fig. 2. In fact, the identical regions flanking the 5' ends of the two copies of the 23S rRNA gene appear to extend several hundred bases beyond nucleotide 1, as seen by comparison of the patterns on the sequencing gel (data not shown). The nucleotide sequences within the inserts of p54X1.1 and pB1X1.1 begin to diverge from position 63 downstream of the 5S rRNA gene (Fig. 3).

Three Pribnow boxes similar to the consensus sequence TA TAAT were identified at the beginning of the *H. pylori* 23S gene (Fig. 2). The consensus sequence (TTGAC) of the -35 region could not be clearly defined upstream of any of the predicted Pribnow boxes. A putative end of the 23S rRNA gene was proposed at position 3339 (Fig. 2) on the basis of sequence alignment of 23S rRNA genes from a number of other prokaryotes with the sequence obtained here (data not shown). The 5' terminus of the mature 23S rRNA gene transcript was determined by primer extension (Fig. 4). The 5'-terminal nucleotide appears to be either A or T (Fig. 4), which corresponds to position 372 (T) or 373 (A) in Fig. 2. Since the majority of the primer extension product was generated at position 373, we define the 5' terminus of the mature 23S rRNA as position 373. Therefore, the complete sequence of the *H. pylori* 23S gene likely consists of 2,967 bp. In addition, the 5'-end and the putative 3'-terminal nucleotides of the 5S rRNA gene were also determined by primer extension and sequence alignment (21), respectively.

Because the sequences of the two copies of the 23S-5S genes were demonstrated to be identical, we thought it possible that only one copy of the genes was represented by these two clones. At some point during the cloning of the genes some rearrangement might have occurred, giving the appearance that the nucleotide sequences of the two copies diverge from 63 bp to the 3' end of the 5S rRNA genes (Fig. 3). To demonstrate that the two cloned copies of the 23S-5S rRNA genes represent different origins on the chromosome, primers were designed from the sequence of the clones where they were different. These primers, primers 54.ext and B1.ext, were then

used to amplify PCR products from the plasmid clones (clones p54X1.1 and pB1X1.1), lambda clones (clones  $\lambda$ 23.54 and  $\lambda$ B1), and UA802 chromosomal DNA in combination with primer 5h, which was generated from the region spanning positions 66 to 82 from the 3' end of the 23S rRNA gene (Fig. 2). In combination with primer 5h, a product of 655 bp was produced only from the DNA templates p54X1.1,  $\lambda$ 23.54, and the chromosomal DNA with the primer 54.ext, whereas with the primer B1.ext, a product of 569 bp was amplified only from the clones pB1X1.1,  $\lambda$ B1, and the chromosomal DNA (data not shown). If the two copies of the 23S-5S rRNA gene cluster which we identified were generated by rearrangement of a single 23S-5S rRNA gene copy during the cloning process, a different-sized PCR product or no product would be amplified from the chromosomal DNA with either primer 54.ext or primer B1.ext. The results presented here demonstrated that the 23S-5S rRNA genes cloned into p54X1.1 and pB1X1.1 do represent copies of these genes separate from the chromosome.

**Phylogenetic comparisons of known prokaryotic 23S rRNA sequences.** Sequence analyses and phylogenetic comparisons were done by using the Wisconsin Sequence Analysis Package (GCG). The complete sequence of the 23S gene of *H. pylori* UA802 was compared to those of the 23S genes from the prokaryotes *Campylobacter jejuni* (GenBank accession no. U09611), *Campylobacter coli* (GenBank accession no. Z29326), *Burkholderia cepacia* (formerly *Pseudomonas cepacia*; GenBank accession no. 16368), *E. coli* (GenBank accession no. V00331), *Rhodobacter capsulatus* (GenBank accession no. X06585), *Anacystis nidulans* (GenBank accession no. X00512), *Bacillus subtilis* (GenBank accession no. D11460), and *Micrococcus luteus* (GenBank accession no. X06484) by using the Pileup program of the GCG package. The dendrogram constructed from this comparison is presented in Fig. 5. The comparisons indicated that *H. pylori*, along with *C. jejuni* and *C. coli*, belong to a separate subdivision, as proposed by Trust et al. (42).

**MICs of clarithromycin-resistant clinical isolates of *H. pylori*.** The MICs of both clarithromycin and erythromycin were determined for six *H. pylori* isolates which originated from Ireland ( $n = 4$ ), Australia ( $n = 1$ ), and Canada ( $n = 1$ ) (Table 1). Previous studies have demonstrated that all Canadian *H. pylori* strains examined were susceptible to clarithromycin and erythromycin (41). The MICs ranged from 0.002 and 0.06  $\mu$ g/ml (susceptible) to 32 and 256  $\mu$ g/ml (resistant) for clarithromycin and erythromycin, respectively. Intermediate MICs of both antibiotics were found for strain B: 0.5 to 1  $\mu$ g/ml for clarithromycin and 64 to 128  $\mu$ g/ml for erythromycin.

**Natural transformation of clarithromycin resistance determinant(s) between *H. pylori* strains.** Chromosomal DNA isolated from the  $\text{Cla}^{\text{r}}$   $\text{Ery}^{\text{r}}$  *H. pylori* E was used to transform the  $\text{Cla}^{\text{s}}$  strain UA802.  $\text{Cla}^{\text{r}}$  UA802 transformants were selected on BHI-YE agar supplemented with either 2  $\mu$ g of clarithromycin per ml or 32  $\mu$ g of erythromycin per ml. Single colonies, two from clarithromycin selection and three from erythromycin selection, were used for MIC tests. For all  $\text{Cla}^{\text{r}}$  and  $\text{Ery}^{\text{r}}$  UA802 transformants the MICs of both clarithromycin and erythromycin were equal to those for donor strain E (Table 1).

The transformation frequencies of the  $\text{Cla}^{\text{r}}$   $\text{Ery}^{\text{r}}$  determinants from donor strains B and E to the recipient strain, strain UA802, were determined. Transformants were selected by using BHI-YE agar containing 16  $\mu$ g of erythromycin per ml, while the total number of viable cells was determined by using BHI-YE agar without antibiotics (data not shown). All experiments were carried out in triplicate. The transformation frequency of strain B was found to be  $2.4 \times 10^{-6} \pm 0.47 \times 10^{-6}$

1 AAAGCTTCATCCACCCCCCATCCCATCATTTCCAATCACTTTTATCCATTTCTTCAAACCCAAAACTTTAAGCAAAGTTAAGCATGTCTATAATF  
 101 ACATTTTCGTTTTAAAGACAAGCTTTAAAAGTCTTTAATGAACCACTCAACAAGTTCTACAAGCTAAAGCTTTAAATAAAACCCACCAGCTGGTAAAAAC  
 201 TTGAGTGTATAAAAAGATTAGGGATCAAGCATTTTCTAGTCTTCTTAAAGGGTTAAACATTAAGAGTGATTATAGCAAGTTTTTAAAGAAAAACGAAGTT  
 301 ATTTGATTTAACATTGTTAATAGCCTATGTAAGTAAAGTAAACTACAATAACTCTGTCTTATATTCATTAAAGGCAGTGGTAGCGCTGAAGAATGTTTC  
 401 GTGCAATTGTCGTATTCATTATAAAAGGGCGGGTTTTAAAGGATATTTTAAATTTAAAACAAGCTTTTAAAGAGCAGATGGCGGATGCCTTGCCAAAGA  
 501 GAGGCGATGAAGGACCTACTAGACTGCGATAAGCTATGCGGAGCTGTCAAGGAGCTTTGATGCGTAGATGTCGGAATGGGGCAACCCAACTAATAGAGAT  
 601 ATTAGTTACTCTAACAGAGAGCGAACCTAGTGAAGTAAAACATCTCAGTAACTAGAGGAAAAGAAATCAACGAGATCCCTAAGTAGTGGCGAGCGAACG  
 701 GGGAAAAGGGCAAACCGAGTGTTCATTGCGGGTTGAGGACTGCAACATCCAAGAGAACCGCTTTAGCAGAGTTACCTGAAAAGGTAAAGCCATAGAAAGT  
 801 GATAGCCTTGATGCGACAAGGCGTTTTAGGTAGCAGTATCCAGAGTAGGCCAGGACAGGGAATCCAGGTTGAAGCCGGGAGACCCTCTCCAACF  
 901 CTAATACTACTCTTTGAGCGATAGCGAACAGTACCCTGAGGGAAAAGGTGAAAAGAACCAGTGTAGCGGAGTGAATAGAACCTGAAACCATCTGCTT  
 1001 ACAATCATTCAGAGCCATATGATTTATCAGGGTGTGGACTGCTTTTGCATAATGATCCTGCGAGTTGTGTATCTGCGAAGTTAAGCGAATGCGAAG  
 1101 CCGTAGCGAAACGAGTTCTTAATAGGGCGAACAGTCAAGTGTGACAGCCGAAAGCTAAGTGTATCTATCCATGGCCAAGTTGAAACCGGTGTAATAGCG  
 1201 CGTGGAGGACTGAACTCCTACCCATTGAAACGGGTTGGGATGAGCTGTGGATAGGGGTGAAAGGCCAAACAACTTAGTGATAGCTGGTCTCTCGAAA  
 1301 TATATTTAGGTATAGCCTCAAGTGATAATAAAAGGGGTAGAGCGCTGATTGGGCTAGGGCTGCTCGCCGCGTACCAAACCTATCAAACCTCGAATAC  
 1401 CTTTTATCGTATCTGGGAGTCAGGCGGTGGGTGATAAAATCAATCGTCAAAAGGGGAACAACCCAGACTACCAAATAAGGTCCCTAAGTTCTATTCTGA  
 1501 GTGGAAAAGATGTGTGGCTACTAAAACAACCCAGGAGTTGGCTTAGAAGCAGCCATCCTTTAAAGAAAGCGTAACAGCTCACTGGTCTAGTGGTCATGC  
 1601 GCTGAAAATATAACGGGGCTAAGATAGACACCGAATTTGTAGATTGTGTTAAACACAGTGGTGAAGAGCGTTCATACCAGCGTTGAAGGTATACCGGTA  
 1701 AGGAGTGTGGAGCGGTATGAAGTGAAGTGCAGGAATGAGTAACGATAAGATATATGAGAATTGTATCCGCGTAAATCTAAGGTTTCTACGCGATGG  
 1801 TCCTCATCGTAGGGTTAGTCGGGCTCCTAAGCCGAGTCCGAAAGGGGTAGGTGATGGCAAATGGTTAATATCCAATACCAGCTGTGGAGCGTGTGGGG  
 1901 GGACGCATAGGGTTAAGCGAGCTAGCTGATGGAAGCGCTAGTCTAAGGGCGTAGATTGGAGGGAAGCAATCCACCTCTGTATTTGAAACCCAAACAGG  
 2001 CTCTTTGAGTCCCTTTAGGACAAAGGGAGAATCGTGATACCCTCGTGCCAAAGAAAGCCTCTAAGCATATCCATAGTCGTCCTACCGCAAACCGACAC  
 2101 AGGTAGATGAGATGAGTATTTCTAAGGCGCGTGAAGAAGTCTGGTTAAGGAAGTCTGCAAACTAGCACCGTAAGTTCGCGATAAGGTGTGCCACAGCGAT  
 2201 GTGGTCTCAGCAAAGAGTCCCTCCCGACTGTTACCAAAAACACAGCACTTTGCCAACTCGTAAGAGGAAGTATAAGGTGTGACGCTGCCCGGTGCTCG  
 2301 AAGTTAAGAGGATGCGTCAGTCGCAAGATGAAGCGTTGAATTGAAGCCCGAGTAAACGGCGCGTAACTATAACGGTCTAAGGTAGCGAAATTCCTT  
 2401 GTCGGTTAAATACCGACCTGCATGAATGGCGTAACGAGATGGGAGCTGTCTCAACCAGAGATTCAAGTGAATTTAGTGGAGGTGAAAATTCCTCTTACC  
 2501 CGCGCAAGACGGAAAGACCCCGTGGACCTTACTACAACCTAGCACTGCTAATGGGAATATCATGCGCAGGATAGGTGGGAGGCTTTGAAGTAAGGGCT  
 2601 TTGGCTCTTATGGAGTCATCCTTGAGATACCACCTTGATGTTTCTGTTAGCTAAGTGGCTGTGTTATCCACAGGCAGGACAATGCTTGGTGGGTAGTT  
 2701 TGACTGGGGCGTTCGCTCCTAAAAGTAAACGGAGGCTTGCAAAGGTTGGCTCATTGCGGTTGGAAATCGCAAGTTGAGTGAATGGCACAAGCCAGCCTG  
 2801 ACTGTAAGACATACAAGTCAAGCAGAGACGAAAGTCCGTCATAGTGTGTCGGTGGTCTGTGTGGAAGGGCCATCGCTCAAAGGATAAAAGGTACCCCGG  
 2901 GGATAACAGGCTGATCTCCCCAAGAGCTCACATCGACGGGAGGTTGGCACCTCGATGTCGGCTCATCGCATCCTGGGGCTGGAGCAGGTCCCAAGGG  
 3001 TATGGCTGTTTCGCCATTTAAAGCGGTACGCGAGCTGGGTTCAGAACGTCGTGAGACAGTTTCGGTCCCTATCTGCCGTGGGCGTAGGAAAGTTGAGGAGAG  
 3101 CTGTCCCTAGTACGAGAGGACCGGGATGGACGTGCTCACTGGTGCACAGTTGTCTGCCAAGAGCATCGCTGGGTAGCACACCGGATGTGATAACTGCTG  
 3201 AAAGCATCTAAGCAGGAACCAACTCCAAGATAAACTTTCCCTGAAGTTCGCACAAAGACTATGTGCTTGATAGGGTATAGTGTGAGCGCAGTAATGCGT  
 3301 TTAGCTGACTACTAATAAGAGCGTTGGCTTGTGTTTTGCTTTTTGATAAGATAACGGCAATAAGCGCAATGGGTTACCCTGCTTACTGAGTGTGA  
 3401 AGAGAGTTGGAGTTTTATGAAGACTTTTATAAGATTAACCTTTAATGAGGAATGAGATACCATCTCAATGGTTTAAAGTTAAAGGCTAATTAACGATCTTC  
 3501 TTTGTTAAAACAGCTCCCTATAAAGAGAAAGGGGAGTTAAGGTTAAATGCGTTTTTATCTTTAGCTCCCTTTCCCTGTGCTTTAGAGAAGAGGAAC  
 3601 TACCAGTTAACCATCCGAACCTGGAAGTCAAGCTCTTCATCGCTGATAAATGCTCTTTTCAAGAGTGGGAATGTAGGTCGTCAGGGATAGGGAA  
 3701 ATGTTTTTTTAGTCTTGCTTTTTTATTTGATTTTATTGACTCATTTGTTTTGTTGTTAGG

FIG. 2. Complete nucleotide sequence of the 23S and 5S rRNA genes as well as part of the flanking region from clones p54X1.1 and pB1X1.1. Putative transcription elements (Pribnow boxes; underlined) and the 5' and 3' ends of the 23S and 5S rRNA genes (in boldface type) are indicated. The nucleotide denoted by an asterisk represents an alternative 5' terminus of the 23S rRNA. The regions from which individual primers were generated are denoted. Primer sequences are oriented by arrows from 5' to 3'; primers PEP, DP4, DP5, and ZGE23 are complementary to the nucleotide sequence, whereas primers 5g, DP1, and 5h are identical to the indicated sequence. The 3'-terminal residue C of either DP4 or DP5 is complementary to G at either positions 2142 or 2143 of the 23S rRNA from *Cla*<sup>I</sup> *H. pylori* isolates.

**p54X1.1** TTGTTTGTTTAGGGTTTAAATGTTTTATGTTTTATTCAAAGGTTTTAGGGTTTAAAGAA  
**pB1X1.1** TTGTTTGTTTAGGTGGTTTTATGTTGGGTTTTGGTTGTTTTGTTGATTTAGTTTTGCATGCTC

**p54X1.1** AACGAGTTTGA AAAACAAAATCTAACATAATAAAAATAAAACTTAAAATCCAATTTTTAAA  
**pB1X1.1** TAAACCGATGAAAGGTTGTTTGAAGTCTTCTCTGTTTCATGGGGCTTGCTAATAACGCATC  
 ←

**p54X1.1** AAGTTTAAGATTTTATATCTTTGGTAGAAAGAGTCCGTATCA  
 ← **54.ext**  
**pB1X1.1** TCTTCTCTAC

FIG. 3. Sequence of DNA downstream of the 5S rRNA gene from the points where p54X1.1 and pB1X1.1 diverge. Residues in boldface type represent the end of the sequence up to where the sequences of the two clones are identical. Underlined sequences indicate regions from which the primers were generated.

per viable cell, and that for strain E was found to be  $1.9 \times 10^{-6} \pm 0.47 \times 10^{-6}$  per viable cell.

**Association of the *H. pylori* 23S gene with clarithromycin and erythromycin resistance.** To investigate whether the restriction digestion profiles of the genomes within the five *Cla*<sup>r</sup> Ery<sup>r</sup> UA802 mutants were altered in comparison with that for the parental strain, their chromosomal DNAs were digested with *Not*I and separated by pulsed-field gel electrophoresis. Significant differences in such restriction profiles between the *Cla*<sup>r</sup> Ery<sup>r</sup> mutants of UA802 and the wild-type strain were found for only one mutant, strain UA802 *Cla*<sup>r</sup>-2 (data not shown). Previous studies by Southern hybridization with the 23S rRNA gene probe indicated that two copies of the 23S rRNA gene resided within the *Not*I fragments 1 and 3 derived from wild-type UA802 (22). Examination of the mutants

Ery<sup>r</sup>-1, Ery<sup>r</sup>-2, Ery<sup>r</sup>-4, and *Cla*<sup>r</sup>-1 showed no changes in the location of the 23S rRNA genes. In contrast, in the mutant *Cla*<sup>r</sup>-2 chromosome, a 23S rRNA copy was detected on *Not*I fragment 5. To determine if any mutations within the 23S rRNA gene of these transformants occurred at positions cognate with *E. coli* 23S rRNA gene, A2058 and A2059, DNA fragments were amplified from the respective *Cla*<sup>r</sup> transformant chromosomal DNAs by using PCR with primers 5g and

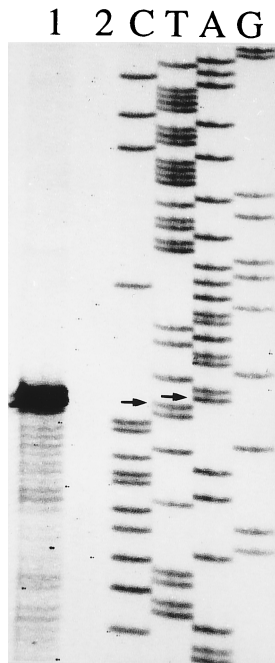


FIG. 4. Primer extension analysis. The procedures for RNA isolation and primer extension with primer PEP are detailed in the Materials and Methods section. Lanes C, T, A, and G, dideoxy-terminated sequence by using the same primer and p54X1.1 DNA as a template; lane 1, RNA minus RNase; lane 2, RNA plus RNase. Nucleotides complementary to the transcription start site (A or T) were denoted by arrows.

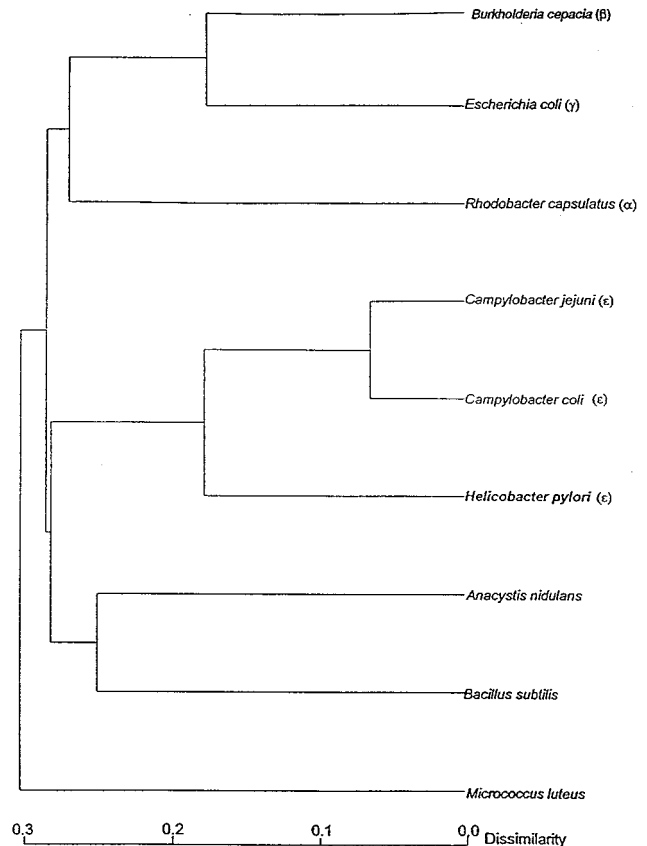


FIG. 5. Phylogenetic tree based on comparisons of complete 23S rRNA gene sequences, including those of representative organisms from four of the *Proteobacteria* subdivisions and representative organisms of the phyla of gram-positive bacteria (*B. subtilis* and *M. luteus*) and cyanobacteria (*A. nidulans*). The *Proteobacteria* subdivisions are indicated in parentheses.

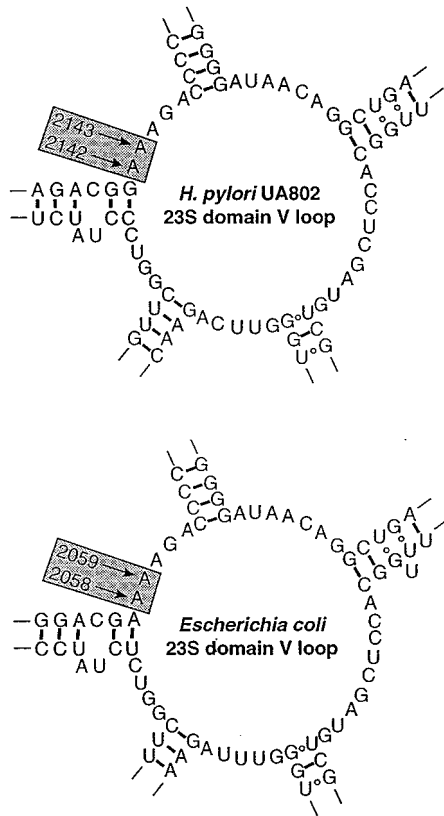


FIG. 6. Comparison of the domain V loops of the 23S rRNA molecules from *H. pylori* UA802 and *E. coli* according to the model of Noller (25). The loop of the *E. coli* 23S rRNA is modified from that drawn by Egebjerg et al. (11). Positions associated with clarithromycin and erythromycin resistance in the respective 23S rRNA molecules are indicated by numbers and arrows.

ZGE23 (Fig. 2) and were sequenced. If only one copy of the 23S rRNA gene was mutated, equal amounts of PCR products from the mutated and wild-type copies should be amplified. Therefore, both the mutated nucleotide (G) and the wild-type nucleotide (A) should be revealed on the sequencing gel. However, only nucleotide G at position 2142 in the 23S rRNA gene was identified, demonstrating that both copies of the 23S rRNA present in  $\text{Cl}^{\text{r}}$   $\text{Ery}^{\text{r}}$  strains have been mutated as an A-to-G transition (Fig. 6).

**PCR detection of point mutations of the 23S rRNA genes.** The detection of point mutations in *H. pylori* by using 3' mismatched PCR primers has been described previously (17); therefore, PCR primers were designed to detect the A-to-G transition mutation in the 23S rRNA genes of *H. pylori*. Primers 5g (5'-CTCAGGGTGATGGACTG-3') and ZGE23 served as positive controls, and primer 5g was used in combination with DP4 (5'-AGGTCCACGGGGTCTTC-3') or DP5 (5'-AAGGTCCACGGGGTCTTC-3') to detect the mutation at positions 2142 or 2143, respectively (Fig. 2). The reactions with 5g-DP4 and 5g-DP5 produced amplicons of 1,505 and 1,506 bp, respectively. The PCR mixtures contained 1.0 mM  $\text{MgCl}_2$  and 50  $\mu\text{M}$  deoxynucleoside triphosphates and were cycled at 94°C for 1 min, 57°C for 20 s, and 72°C for 1 min for 35 cycles. The lack of a PCR product indicated that the transition mutation was not present in either copy of the 23S rRNA gene. Strain UA802 produced an amplicon of 1.6 kb with only the control primers 5g-ZGE23 but no amplicon with the 3' mismatched primers, either 5g-DP4 or 5g-DP5 (data not shown).

In contrast, all of the transformed strains produced an expected product with both the control and the 3' mismatched primers (5g-DP4), whereas they did not give rise to a PCR product with the 3' mismatched primers (5g-DP5) (data not shown). These results demonstrate that an A-to-G mutation at position 2142 occurred in the 23S rRNA gene of all the  $\text{Cl}^{\text{r}}$  transformants tested, which confirms the sequencing results presented above. Primers 5g and DP5 were able to detect the mutation at position 2143 in *H. pylori* B, a change which was found previously when the region was sequenced. Such primer sets may be used as part of a screening method for the detection of point mutations conferring resistance to the macrolide group of antibiotics.

## DISCUSSION

Early studies of the genomic physical and genetic maps of various *H. pylori* strains have demonstrated that the *H. pylori* genome contains two copies of the 23S rRNA gene (3, 22). In this study, we have demonstrated that the nucleotide sequences of the two copies of the 23S rRNA gene from *H. pylori* UA802 are identical. In addition, a 5S rRNA gene is linked to the 3' ends of these two 23S rRNA gene copies. It is not surprising that the sequences of both copies of the 23S and 5S rRNA genes as well as the flanking and intervening regions within *H. pylori* UA802 are identical. In *B. subtilis* (19, 28), all six copies of the 23S and 5S rRNA genes were found to be almost identical, with only minor variations in two copies (*rrnJ* and *rrnW*); the 5'-flanking regions of the 23S rRNA genes for the *rrnO* and *rrnB* are identical for at least 327 bp, as are the 5'-flanking regions of *rrnA* and *rrnI*.

The nucleotide sequence of the 5' region of the 23S genes of *H. pylori* UA802 did not reveal the presence of a 16S rRNA gene (Fig. 2), indicating that the *H. pylori* 16S rRNA gene is not linked to the 23S-5S gene cluster in either copy in this particular strain. This finding is consistent with results obtained from the construction of genomic restriction maps of several different *H. pylori* strains (3, 22, 39, 40). Although the rRNA genes of prokaryotes are generally clustered together in operons, separation of these genes occurs within certain organisms such as some strains of *Borrelia* spp. (34), *C. jejuni* and *C. coli* (24, 39), *Mycoplasma hyopneumoniae* (38), *Mycoplasma gallisepticum* (5), *Leptospira interrogans* (14), *Pirellula marina* (23), *Thermus thermophilus* (20), and *Thermoplasma acidophilum* (31).

Three Pribnow boxes were predicted from the region upstream of the *H. pylori* 23S rRNA gene. The Pribnow box, designated P1 (Fig. 2), is 4 or 5 bp from the 5'-terminal nucleotide T or A of the mature 23S rRNA molecule, determined by primer extension (Fig. 4). Therefore, it is likely that these two terminal nucleotides represent the transcription start sites of the 23S rRNA gene, suggesting that the 5' end of the *H. pylori* 23S-5S gene transcript is not posttranscriptionally processed. In contrast, transcription of the rRNA gene operons in *E. coli* begins almost 300 bp upstream from the 16S rRNA gene (8), which is regulated by two tandem promoters (25). The individual rRNA molecules are then generated by posttranscriptional processing.

Two positions associated with clarithromycin and erythromycin resistance in the *H. pylori* 23S rRNA were inconsistently numbered by different groups of investigators and included A2058 and A2059 (43, 44), A2514 and A2515 (7), and A2143 (35-37). In this study, the 5' end of the *H. pylori* 23S rRNA was determined by primer extension as nucleotide A corresponding to position 373 in Fig. 2, as discussed above. Therefore, to avoid further confusion, we propose that positions associated



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