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Nucleotide Sequence of the *gyrA* Gene and Characterization of Ciprofloxacin-Resistant Mutants of *Helicobacter pylori*

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PCR was used to amplify a 238-bp region from *Helicobacter pylori* which corresponded to the quinolone resistance-determining region in *Escherichia coli*. The *gyrA* gene of *H. pylori* was cloned and sequenced. An open reading frame of 2,478 nucleotides coded for a polypeptide of 826 amino acids with a calculated molecular mass of 92,508 Da. The amino acid sequence showed an overall 52% identity with other bacterial *gyrA* genes but was most closely related to the *gyrA* subunit of *Campylobacter jejuni* (76.5% identity). Sequencing of the amplification product from ciprofloxacin-resistant mutants of *H. pylori* revealed four classes of mutations with substitutions at amino acid 87 (Asn→Lys), amino acid 88 (Ala→Val), and amino acid 91 (Asp→Gly, →Asn, or →Tyr) and a double substitution at amino acids 91 and 97 (Ala→Val). Ciprofloxacin-susceptible strains of *H. pylori* could be transformed to ciprofloxacin resistance by using the amplified fragment from resistant strains as donor DNA. Of the 11 ciprofloxacin-resistant mutants examined, only one did not have an alteration within the quinolone resistance-determining region, suggesting that, in *H. pylori*, resistance to quinolones is primarily a result of alterations in *gyrA*.

Considerable evidence implicating *Helicobacter pylori* as an essential etiologic agent in type B chronic gastritis and peptic ulcer disease exists (2, 19, 20, 37). Currently, *H. pylori* infections are treated with two or three antimicrobial agent combinations, as single therapy has proven ineffective (19, 21). As new approaches for antimicrobial agent treatment of *H. pylori* are developed, it is increasingly important to identify potential resistance problems. We have detected resistant *H. pylori* isolated from patients treated with ciprofloxacin, an antimicrobial agent which has potent in vitro activity, and therefore undertook an examination of such strains to determine their mechanism of resistance. Ciprofloxacin exerts antimicrobial activity by inhibiting the enzyme DNA gyrase (26). This enzyme, in addition to relaxing supercoiled DNA, is able to introduce negative supercoils into DNA and thus maintains the bacterial chromosome in a negatively supercoiled state (26). In addition, the enzyme is involved in DNA replication, recombination, and transcription (13, 32). The bacterial enzyme is a tetramer consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. In *Bacillus subtilis* (23), *Staphylococcus aureus* (12, 18), and *Mycoplasma pneumoniae* (4) these genes are contiguous on the chromosome, while in *Escherichia coli* (30), *Klebsiella pneumoniae* (6), *Campylobacter jejuni* (34), and *Pseudomonas aeruginosa* (14) the genes are located separately. As an enzyme essential for cell replication, DNA gyrase is an obvious target for antimicrobial agents. The fluoroquinolones exert their antimicrobial activity at the level of the A subunit of the DNA gyrase. This subunit, responsible for DNA cleavage and rejoining, is also the site of action for nalidixic acid. In *E. coli*, mutations in the *gyrA* gene which result in high-level resistance to quinolones fall within amino acids 67 to 106 of the *gyrA* subunit (5). This region has been termed the "quinolone resistance-determining region" (QRDR) (36) and involves amino acid substitution near the active-site tyrosine (amino acid 122 in *E. coli*). In other bacteria as well, resistance to quinolones has been attributed to specific mutations in *gyrA* (29, 33) and occasionally to mutations in *gyrB* (35). In this study we have identified mutations in ciprofloxacin-resistant isolates of *H. pylori* which are responsible for quinolone resistance. In addition, we report the cloning and nucleotide sequence of the *gyrA* gene of *H. pylori.*

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Clinical isolates of *H. pylori* used in this study were obtained from Foothills Hospital, University of Calgary, Calgary, Canada. Isolates were obtained from stomach biopsies from patients enrolled in a clinical trial which examined the efficacy of ciprofloxacin in treating *Helicobacter* infections. Isogenic pairs of ciprofloxacin-susceptible and -resistant *H. pylori* isolates were obtained by isolating bacteria from patients pre- and post-ciprofloxacin treatment and were determined to be the same strains by using a restriction digest analysis system (22). *Helicobacter* strains were grown on charcoal agar plates (Difco, Ann Arbor, Mich.) supplemented with 5% heat-inactivated horse serum and containing vancomycin (10 mg/liter), polymyxin B (2,500 U/liter), and trimethoprim (5 mg/liter). Plates were incubated at 37°C in a converted anaerobic chamber containing 5% O_2, 10% CO_2, and 85% N_2 or in anaerobic jars containing 10% CO_2. Ciprofloxacin-resistant strains were identified by using E tests (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions on charcoal agar.

MICs were determined via agar dilution as previously described (7) on Brucella broth agar (Difco) containing 7% lysed horse blood.

DNA isolation and nucleotide sequence analysis. Chromosomal DNA was isolated from *H. pylori* as previously described (17). Nucleotide and amino acid sequence data were analyzed with an IBI Pustell sequence analysis program. GenBank searches and dendrogram production used PC Gene nucleic acid and protein sequence software (Intelligenetics, Inc., Mountain View, Calif.).

Construction of an *H. pylori* genomic library. A gene library was constructed as follows. Chromosomal DNA from *H. pylori* UC946 was partially digested with *Sau*3A and fractionated on a 10 to 40% sucrose gradient, and 20- to 40-kb fragments were collected. Fragments were ligated into *Bam*HI-cut cosmid vector pHC79 (11), and the ligation mix was packaged into lambda (Promega Corp., Madison, Wis.) and transduced into *E. coli* HB101. Cells were then plated onto Luria broth plates containing ampicillin (50 μg/ml). After overnight incubation at 37°C, ampicillin-resistant colonies were pooled and stored at −70°C in Luria broth containing 10% glycerol.

Colony hybridization. Colony hybridization for identifying cosmid clones was performed on Hybond nylon membranes (Amersham) under high-stringency conditions (65°C, 1% sodium dodecyl sulfate [SDS], 10% dextran sulfate, and 1 M NaCl) by the method described by Sambrook et al. (28). Radioactive labeling

of PCR products was done with a random primer oligonucleotide labeling kit (Pharmacia, Baie d'Urfé, Canada).

PCR. A mixed set of degenerative primers designed from conserved regions of the *E. coli gyrA* gene were used to initially amplify a ca. 270-bp region from the *gyrA* gene of *H. pylori* UC763. Primer 38+HPGyr corresponded to amino acids 38 to 46 of the *E. coli gyrA* gene [5′-CGAGA(TC)GG(IA)CTIAA(AG)CC (IA)GT(IC)CA(TC)(AC)G], and primer 127-HPGyr corresponded to amino acids 119 to 127 [5′-CAT(CT)G(GT)IA(CT)(CT)TC(IA)GT(GA)TA(IC) C(GT)CAT(CI)GC]. These primers were used in a "touchdown" PCR protocol (8) in reaction mixtures containing various concentrations of template chromosomal DNA. Optimal results were obtained with 6.5 ng of template DNA with the following thermocycling parameters: 97°C for 10 min; eight cycles of 94°C for 1.5 min, 55°C for 0.5 min, and 72°C for 1 min; eight cycles of 94°C for 1.5 min, 54°C for 0.5 min, and 72°C for 1 min; eight cycles of 94°C for 1.5 min, 53°C for 0.5 min, and 72°C for 1 min; eight cycles of 94°C for 1.5 min, 52°C for 0.5 min, and 72°C for 1 min; eight cycles of 94°C for 1.5 min, 51°C for 0.5 min, and 72°C for 1 min; and 72°C for 5 min. A small amount of the amplification product was thus obtained and purified by using a Magic Prep DNA purification kit (Promega). This DNA was reamplified with a single pair of primers chosen at random from the 38+HPgyr and the 1127-HPgyr mixed primers. These primers were 39+GyrHPsolo (5′-GATGGA CTCAAGCCAGTCCATAG) and 127-GyrHPsolo (5′-CATCGGCATCTC AGTGTACCTCA). The PCR was carried out by using the touchdown parameters, and the amplified DNA was purified with the Magic Prep kit and sequenced by using a Circumvent thermal dideoxy DNA sequencing kit (New England BioLabs, Mississauga, Canada) according to the instructions of the manufacturer. The sequence data were used to design two primers internal to 39+GyrHPsolo and 127-GyrHPsolo, designated GyrHPfor (5′-AATTAGGCCTTACTTCCAAAGTCGCTTACA) and GyrHPrev (5′-TCT TCACTCGCCTTAGTCATTCTGGC), respectively. These primers were used to amplify a 238-bp region representing the QRDR from all other *H. pylori* strains used in this study. Reactions requiring the GyrHPfor and the GyrHPrev primers were performed by using the following thermocycling program: 94°C for 3 min and 30 cycles of 94°C for 1 min, 55°C for 0.5 min, and 72°C for 1.5 min. Amplifications were done in a 50-μl reaction volume containing 25 to 35 pmol of the oligonucleotide primer; 200 μm (each) dATP, dCTP, dGTP, and dTTP (Stratagene, La Jolla, Calif.); reaction buffer (Bethesda Research Laboratories, Burlington, Canada); 10 μl of a template DNA sample (containing between 10 and 100 ng of DNA); and 2.5 U of *Taq* polymerase (Bethesda Research Laboratories). When required, reaction products were visualized by running 5 μl of the reaction mixture on a 1.2% agarose gel. PCR sequencing of the amplified DNAs from various strains of *H. pylori* examined in this study was accomplished by using the Circumvent kit.

Transformation of *H. pylori*. Transformation of *H. pylori* was accomplished by using a modified version of the method described by Wang et al. (34). *H. pylori* HP165, a clinical isolate, was chosen as a recipient for transformation experiments on the basis of prior experiments which revealed that it was readily transformed with DNA from other strains of *H. pylori*. The recipient strain was grown for 2 to 3 days on charcoal agar and then subcultured to a new plate in a 1-cm^2 area. The plate was incubated overnight, donor DNA (1 to 10 ng previously purified with the Magic Prep kit) in a volume of 10 μl was applied to the cells, and the plate was incubated for a further 24 h. The cells were then scraped off the plate into phosphate-buffered saline, pH 7.2, diluted, and plated onto charcoal agar containing 4 μg of ciprofloxacin per ml. Colonies appearing after 2 to 3 days were counted, and four or five colonies from each transformation were purified and maintained on media containing ciprofloxacin. When required, donor DNA was treated with DNase (Sigma) at a final concentration of 2.5 μg/ml for 1 h at 37°C.

Nucleotide sequence accession number. The nucleotide sequence reported here appears in the Genome Sequence Database under the accession number L29481.

RESULTS

Amplification of the QRDR from the *gyrA* gene of *H. pylori*. By using the PCR protocols described above, a 238-bp DNA fragment from *H. pylori* UC763 chromosomal DNA was amplified. To confirm that the amplification product was from the *gyrA* gene, it was sequenced and compared with known *gyrA* sequences from other bacteria. The amplified product from *H. pylori* displayed a 91% overall identity with *gyrA* sequences from *E. coli* (30), *K. pneumoniae* (6), *S. aureus* (29), *C. jejuni* (33), and *P. aeruginosa* (14) (Fig. 1). These results indicate that the 238-bp PCR product was from the *H. pylori gyrA* gene. The amplified product corresponded to the QRDR of the *E. coli gyrA* gene.

Cloning the *H. pylori gyrA* gene. The *H. pylori* genomic library was screened by colony hybridization using a ^{32}P-labeled 238-bp PCR-amplified fragment as a probe. Three recombinant clones containing DNA homologous to the probe were identified. One clone, pHP40, contained an insert of approximately 40 kb of *H. pylori* chromosomal DNA. This plasmid was digested with *Eco*RI, and a 10-kb fragment was subcloned into pUC19. The resulting subclone, p19-10, hybridized with the 238-bp probe and was used as a template for nucleotide sequencing of the *gyrA* gene. Another subclone, p19-4, was generated by digesting p19-10 with *Xba*I and religating the mixture into the *Xba*I site of the expression vector pGEM7Zf(+) (Promega).

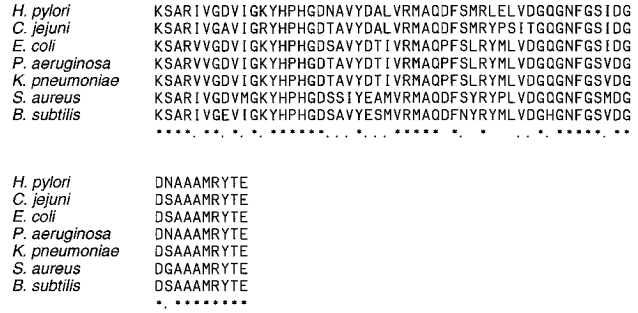

FIG. 1. Comparison of the amino acid sequences of the QRDRs of seven *gyrA* genes. Perfectly conserved (*) and well-conserved (.) positions are indicated. The *E. coli* sequence represents amino acids 65 to 124 of the *gyrA* polypeptide.

Nucleotide sequence of the *H. pylori gyrA* gene. Initial *gyrA* sequence information was obtained via PCR sequencing of the 238-bp *gyrA* fragment amplified from *H. pylori* UC763. This sequence information was used to design primers for dideoxy-chain termination reactions using double-stranded, CsCl-purified p19-10 DNA as the template. Opposite-strand sequencing was performed as described above and by using an Applied Biosystems automated sequencer (University Core DNA Services, University of Calgary). The nucleotide sequence and predicted amino acid sequence of the *H. pylori gyrA* gene are shown in Fig. 2. An open reading frame of 2,478 bp coding for a polypeptide of 826 amino acids with a calculated molecular mass of 92,508 Da was found. The DNA had a G+C content of 41.4%, which is slightly higher than the G+C content (36 to 37%) of the entire *H. pylori* chromosome (1).

Sequence analysis revealed a ribosome-binding site with homology to the *E. coli* Shine-Dalgarno consensus sequence located 4 nucleotides upstream from the ATG start codon. Upstream of the Shine-Dalgarno sequence was a region similar to the −10 *E. coli* promoter consensus sequences TATAAT. Further upstream a potential −35 region was observed (Fig. 2). The amino acid sequence of the *H. pylori gyrA* gene product showed 52.4% overall identity with all other sequenced bacterial *gyrA* peptides. There was 65.9% identity with *E. coli*, 64.7% identity with *K. pneumoniae*, 66.1% identity with *B. subtilis*, 64.7% identity with *P. aeruginosa*, and 76.5% identity with *C. jejuni*. Compared with *E. coli*, the *Helicobacter* gyrase peptide contained an additional region of 45 amino acids starting at amino acid 607 of the *Helicobacter* peptide. An additional region of amino acids was also found in the same area of the *gyrA* peptide of *C. jejuni* (33) and *P. aeruginosa* (14). This region in *H. pylori* displayed only 2% overall homology with the corresponding region in *P. aeruginosa* but showed 75.5% overall homology with the same region in *C. jejuni*. The dendrogram in Fig. 3 illustrates the relatedness of the compared gyrases.

Amino acid changes within the *H. pylori* GyrA gene resulting in ciprofloxacin resistance. The 238-bp region of the *H. pylori*

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TTTCTTTCAATCTTACAAAATAAGCCATGAGTGGATTTCAAAGTCCCCCCCCCAAAGCTAAAGGGTTTAAACGCCTTTT   78

TAAAGCCCTTTTTTATTCTAAAGGCGGGCTTAAATGTGCATGGGTTGAAGAGAGCGCGTTCAGGCAAATTATTATCGT  156

GGCTCTTTGTTGTATCACTCTAGCAAGCTATCTAGCTAAAGATTTTTTAGAATGGGGGCTATTGATTGCGCCTTGTTT  234

TTTATCGGTGGTGGTTGAACTCATCAATAGCTCTATTGAAAAGGCTGTGGATTTTACTGGCACCGAGTTCCACCCTTT  312

                                                          -35
AGCTAAAAAGGCTAAGGACATGGCCAGTTCAGCCCAACTGATAGGGCTTATTTTTTGGGCTTTGATTTGGGGGCGTTA  390

                -10          S-D
TCTTTTAACGCTTTATTTTAATTAAATTTTAGGGAGATACATGCAAGATCGTTTAGTCAATGAAACAAAAAATATTGT  468
                                    M  Q  D  R  L  V  N  E  T  K  N  I  V   13

AGAAGTGGGGATTGATTCTTCTATTGAAGAGAGCTATTTAGCTTATTCAATGAGCGTGATCATAGGGCGCGCTTTACC  546
  E  V  G  I  D  S  S  I  E  E  S  Y  L  A  Y  S  M  S  V  I  I  G  R  A  L  P   39

                                                  GyrHPfor
GGACGCTAGAGATGGCTTAAAGCCCGTGCATAGGCGTATTTTGTATGCGATGCATGAATTAGGTCTTACTTCCAAAGT  624
  D  A  R  D  G  L  K  P  V  H  R  R  I  L  Y  A  M  H  E  L  G  L  T  S  K  V   65

CGCTTATAAAAAAAGCGCTAGGATCGTGGGTGATGTGATTGGTAAATACCACCCCCATGGCGATAACGCGGTTTATGA  702
  A  Y  K  K  S  A  R  I  V  G  D  V  I  G  K  Y  H  P  H  G  D  N  A  V  Y  D   91

TGCACTAGTGAGAATGGCGCAAGATTTTTCCATGCGCTTGGAATTAGTGGATGGGCAGGGCAACTTTGGCTCTATTGA  780
  A  L  V  R  M  A  Q  D  F  S  M  R  L  E  L  V  D  G  Q  G  N  F  G  S  I  D  117

                                                  GyrHPrev
TGGCGATAACGCTGCAGCGATGCGTTACACTGAAGCCAGAATGACTAAGGCGAGTGAAGAAATTTTAAGGGATATTGA  858
  G  D  N  A  A  A  M  R  Y  T  E  A  R  M  T  K  A  S  E  E  I  L  R  D  I  D  143

TAAAGACACCATTGATTTTGTGCCTAATTATGATGACACCTTAAAAGAGCCAGATATTCTACCAAGCCGTCTGCCTAA  936
  K  D  T  I  D  F  V  P  N  Y  D  D  T  L  K  E  P  D  I  L  P  S  R  L  P  N  169

CCTTTTAGTCAATGGGGCTAATGGGATCGCTGTAGGGATGGCGACTTCTATCCCCCCCCACAGAATTGATGAAATCAT 1014
  L  L  V  N  G  A  N  G  I  A  V  G  M  A  T  S  I  P  P  H  R  I  D  E  I  I  195

AGACGCTTTAGCGCATGTCTTAGAAAACCCTAACGCTGAATTAGATGAAATTTTGGAATTTGTCAAAGGGCCTGACTT 1092
  D  A  L  A  H  V  L  E  N  P  N  A  E  L  D  E  I  L  E  F  V  K  G  P  D  F  221

TCCCACTGGTGGGATCATTTATGGCAAGGCGGGCATTATTGAAGCCTATAAAACGGGGCGAGGGCGCGTGAAAGTGCG 1170
  P  T  G  G  I  I  Y  G  K  A  G  I  I  E  A  Y  K  T  G  R  G  R  V  K  V  R  247

GGCCAAAGTGCATGTGGAAAAGACGAAAAATAAAGAAATCATCGTTTTAGATGAAATGCCTTTTCAAACCAATAAAGC 1248
  A  K  V  H  V  E  K  T  K  N  K  E  I  I  V  L  D  E  M  P  F  Q  T  N  K  A  273

CAAATTAGTGGAACAAATCAGCGATTTAGCGCGAGAAAAACAAATTGAAGGCATTAGCGAAGTGCGCGATGAGAGCGA 1326
  K  L  V  E  Q  I  S  D  L  A  R  E  K  Q  I  E  G  I  S  E  V  R  D  E  S  D  299

TAGAGAGGGCATTAGAGTGGTGATTGAATTAAAAAGAGACGCGATGAGTGAAATTGTCTTAAACCACCTTTACAAACT 1404
  R  E  G  I  R  V  V  I  E  L  K  R  D  A  M  S  E  I  V  L  N  H  L  Y  K  L  325

CACCACCATGGAGACCACTTTTAGCATCATTTTACTCGCTATTTACAATAAAGAGCCTAAGATTTTCACGCTTTTAGA 1482
  T  T  M  E  T  T  F  S  I  I  L  L  A  I  Y  N  K  E  P  K  I  F  T  L  L  E  351

GTTGTTGCGCCTTTTCTTAAACCACAGAAAAACCATTATTATAAGACGCACGATTTTTGAATTAGAAAAGGCTAAAGC 1560
  L  L  R  L  F  L  N  H  R  K  T  I  I  I  R  R  T  I  F  E  L  E  K  A  K  A  377

TAGAGCGCATATTTTAGAGGGCTATTTGATCGCCTTGGACAATATTGATGAAATCGTGCAGCTCATTAAAACAAGCCC 1638
  R  A  H  I  L  E  G  Y  L  I  A  L  D  N  I  D  E  I  V  Q  L  I  K  T  S  P  403

AAGCCCAGAAGCGGCTAAAAACGCCTTAATGGAGCGTTTTACTTTGAGCGAGATCCAAAGCAAAGCCATTTTAGAAAT 1716
  S  P  E  A  A  K  N  A  L  M  E  R  F  T  L  S  E  I  Q  S  K  A  I  L  E  M  429

GCGTTTGCAACGCTTAACAGGCCTTGAAAGGGATAAAATCAAAGAAGAATACCAAAACTTGTTGGAGCTTATTGATGA 1794
  R  L  Q  R  L  T  G  L  E  R  D  K  I  K  E  E  Y  Q  N  L  L  E  L  I  D  D  455

TCTCAATGGCATTTTAAAGAGCGAAGATCGCTTGAATGGAGTTGTCAAAACAGAGCTTTTAGAAGTCAAAGAGCAATT 1872
  L  N  G  I  L  K  S  E  D  R  L  N  G  V  V  K  T  E  L  L  E  V  K  E  Q  F  481

TTCTTCTCCAAGGCGCACTGAAATTCAAGAATCTTACGAAAGTATTGACATAGAAGATTTGATCGCTAATGAGCCTAT 1950
  S  S  P  R  R  T  E  I  Q  E  S  Y  E  S  I  D  I  E  D  L  I  A  N  E  P  M  507

GGTGGTGAGCATGAGCTATAAGGGCTATGTGAAAAGAGTGGATTTAAAAGTTTATGAAAAGCAAAATCGTGGCGGTAA 2028
  V  V  S  M  S  Y  K  G  Y  V  K  R  V  D  L  K  V  Y  E  K  Q  N  R  G  G  K  533

GGGCAAGCTTTCAGGCAGCACTTATGAAGACGATTTCATTGAAAACTTTTTGTGGCTAACACGCATGATATTTTGCT 2106
  G  K  L  S  G  S  T  Y  E  D  D  F  I  E  N  F  F  V  A  N  T  H  D  I  L  L  559

CTTTATCACCAATAAGGGGCAATTGTATCATTTGAAAGTCTATAAAATCCCAGAAGCGAGCCGGATCGCTATGGGTAA 2184
  F  I  T  N  K  G  Q  L  Y  H  L  K  V  Y  K  I  P  E  A  S  R  I  A  M  G  K  585

AGCTATTGTGAATTTAATCTCGCTCGCTCCTGATGAAAAGATTATGGCGACTCTAAGCACCAAAGACTTTAGCAATGA 2262
  A  I  V  N  L  I  S  L  A  P  D  E  K  I  M  A  T  L  S  T  K  D  F  S  N  E  611

ACGCTCTTTAGCCTTCTTCACGAAAAATGGTGTGGTGAAGCGCACCAATTTGAGCGAATTTGGAAGCAACAGGAGTTG 2340
  R  S  L  A  F  F  T  K  N  G  V  V  K  R  T  N  L  S  E  F  G  S  N  R  S  C  637

TGGTATCAGAGCGATTGTTTTAGATGAAGGCGATGAATTAGTGAGTGCAAAAGTTGTGGATAAAAACGCTAAGCATTT 2418
  G  I  R  A  I  V  L  D  E  G  D  E  L  V  S  A  K  V  V  D  K  N  A  K  H  L  663

GCTCATCGCATCGCATTTGGGTATTTTCATTAAATTCCCTTTAGAAGATGTGCGCGAGATGGGAAGGAATGCTCGTGG 2496
  L  I  A  S  H  L  G  I  F  I  K  F  P  L  E  D  V  R  E  M  G  R  N  A  R  G  689

GGTTATAGGGATTAGGCTGAATGAAAATGATTTTGTTGTCGGCGCGGTCGTTATTAGCGATGATGGCAACAAGCTTTT 2574
  V  I  G  I  R  L  N  E  N  D  F  V  V  G  A  V  V  I  S  D  D  G  N  K  L  L  715

GAGCGTGAGTGAAAACGGGCTTGGCAAGCAAACTTTAGCCGAAGCGTATAGAGAGCAATCTCGTGGAGGTAAGGGGGT 2652
  S  V  S  E  N  G  L  G  K  Q  T  L  A  E  A  Y  R  E  Q  S  R  G  G  K  G  V  741

CATTGGCATGAAGCTCACTCAAAAAACCGGCAATCTAGTGGGCGTTATCAGCGTGGATGATGAAAATTTGGATTTGAT 2730
  I  G  M  K  L  T  Q  K  T  G  N  L  V  G  V  I  S  V  D  D  E  N  L  D  L  M  767

GATCCTTACTGCAAGCGCAAAAATGATCAGAGTTTCTATTAAAGATATTAGAGAAACCGGAAGAAACGCTAGTGGGGT 2808
  I  L  T  A  S  A  K  M  I  R  V  S  I  K  D  I  R  E  T  G  R  N  A  S  G  V  793

AAAGCTCATAAACACCGCCGATAAAGTCATGTATGTCAATTCTTGCCCTAAAGAAGAAGAGCCAGAAAATTTAGAAAA 2886
  K  L  I  N  T  A  D  K  V  M  Y  V  N  S  C  P  K  E  E  E  P  E  N  L  E  N  819

CTCTCCTACACAATTATTTGAGTGATGCGTNTCTTTTCATTCTTTTATTTTTATTGTATTTTTTAGGGGGGTTCTTTG 2964
  S  P  T  Q  L  F  E  •                                                          826

CATGCTCTAAACCCCCTAGAAGATCAAGAATTTTTAATTTCGGACCGCTTGCAAAATCGTGATCTAGAGTGATGGGCG 3042


AAGAGTATTCCATCTCTAAACCTATTGTCAGGCGCATTAAAACAGACCCCTAGTGTTTTAGG

3104
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FIG. 2. Nucleotide sequence and deduced amino acid sequence of the *H. pylori gyrA* gene. Locations of PCR primers used to amplify the QRDR are indicated. The Shine-Dalgarno (S-D) consensus sequence and the −10 and −35 regions are underlined.

gyrA gene was amplified from four isogenic mutant pairs and seven other ciprofloxacin-resistant isolates. Nucleotide sequencing of the amplification products revealed that amino acid substitutions associated with ciprofloxacin resistance fell into four types (Table 1). The first type was represented by a change at amino acid 87 (Asn→Lys) and was seen in 1 of the 11 resistant isolates. Alignment of the *H. pylori gyrA* nucleotide sequence with the *E. coli gyrA* sequence (30) revealed that this substitution corresponded to amino acid 83 in *E. coli*. The second type, also represented by one isolate, had a substitution at amino acid 88 (Ala→Val) (amino acid 84 in *E. coli*). The third type of substitution, found in 9 of the 11 mutants, was at amino acid 91 (Asp→Gly, →Tyr, or →Asn) (amino acid 87 in

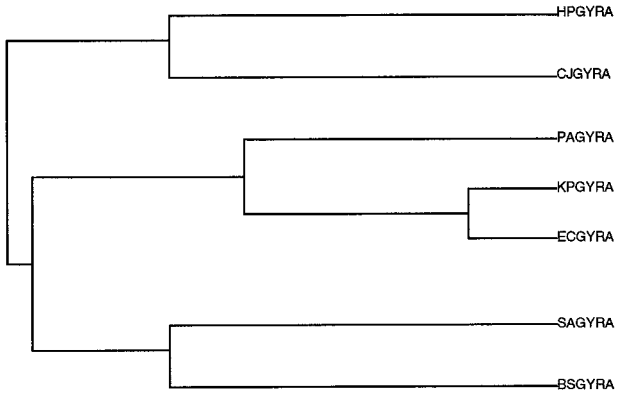

FIG. 3. Dendrogram showing relatedness of seven bacterial DNA *gyrA* peptides. HPGYRA, *H. pylori*; CJGYRA, *C. jejuni*; PAGYRA, *P. aeruginosa*; KP GYRA, *K. pneumoniae*; ECGYRA, *E. coli*; SAGYRA, *S. aureus*; BSGYRA, *B. subtilis*. The dendrogram was derived using the PC Gene program. Degrees of relatedness between different *gyrA* sequences are indicated by the lengths of the horizontal lines (i.e., the shorter the horizontal line, the more similar are the sequences). The maximum horizontal distance has been normalized by the program to equal 1.0.

TABLE 1. Mutations within the *H. pylori gyrA* gene and corresponding amino acid changes which result in ciprofloxacin resistance

Strain and ciprofloxacin MIC (μg/ml)	Mutation	Amino acid change	Comparable change in *E. coli*[a]
Paired[b]			
UC055 (0.25)			
UC742 (4.0)	C→G	Ala-88→Val	Ala-84→Pro
UC414 (0.125)			
UC079 (8.0)	G→A	Asp-91→Asn	Asp-87→Val or →Asn
UC463 (0.0625)			
UC970 (8.0)	C→A	Asn-87→Lys	Ser-83→Ala
UC411 (0.125)			
UC754 (4.0)			
Unpaired			
UC859 (8.0)	A→G	Asp-91→Gly	Asp-87→Val or →Asn
UC151 (8.0)	G→T	Asp-91→Tyr	Asp-87→Val or →Asn
UC357 (4.0)	G→T	Asp-91→Tyr	Asp-87→Val or →Asn
UC602 (8.0)	G→A	Asp-91→Asn	Asp-87→Val or →Asn
UC860 (8.0)	G→A	Asp-91→Asn	Asp-87→Val or →Asn
UC303 (8.0)	G→A	Asp-91→Asn	Asp-87→Val or →Asn
UC561 (8.0)	G→A	Asp-91→Asn	Asp-87→Val or →Asn
	C→T	Ala-97→Val	

[a] Based on comparison of the nucleotide sequence of the *H. pylori gyrA* gene (this study) with the *E. coli gyrA* gene sequence (30).
[b] Isogenic mutant pairs.

TABLE 2. Transformation of *H. pylori* using PCR-amplified *gyrA* DNAs of susceptible and resistant strains

Donor[a]	No. of transformants (CFU)[b]	Mutation in donor	Mutation in transformants[c]
UC463 (S)	1.4×10^{-6}	None	Asp-91→Gly
UC970 (R)	3.6×10^{-4}	Asn-88→Lys	Asn-87→Lys
UC414 (S)	1.5×10^{-6}	None	ND[d]
UC079 (R)	7.3×10^{-4}	Asp-91→Asn	ND

[a] Of a PCR-amplified fragment representing the QRDR of *H. pylori*. Recipient, UC165 (susceptible). See Materials and Methods for the transformation protocol. S, susceptible; R, resistant.

[b] Colonies arising on medium containing 4 μg of ciprofloxacin per ml. The data are averages for three experiments.

[c] At least four transformants from each transformation were examined.

[d] ND, not determined.

E. coli). The fourth type, represented by UC561, had two mutations, one at amino acid 91 (Asp→Asn) and the other at amino acid 97 (Ala→Val) (amino acid 93 in *E. coli*). Finally, a single isolate, UC754, did not have a mutation in the PCR-amplified region of *gyrA*. The location of the mutation(s) conferring resistance in this strain is unknown. Regardless of the type of substitution, the MICs of ciprofloxacin for all of the resistant isolates were similar (4 to 8 μg/ml) (Table 1).

Transformation of ciprofloxacin resistance in *H. pylori*. To establish that the mutations detected in the amplified fragment caused ciprofloxacin resistance, the PCR products were used to transform a ciprofloxacin-susceptible strain of *H. pylori* to a resistant phenotype. The results of these transformation experiments are summarized in Table 2 and demonstrate that the amplified fragment from resistant strains could transform the susceptible recipient to the resistant phenotype at a frequency several hundred-fold higher than the frequency observed when the recipient was transformed with DNA amplified from susceptible cells. Control experiments, in which the recipient received an aliquot of H_2O or DNase-treated DNA, did not yield any resistant colonies (data not shown). To test that the transformed cells contained the mutation present in the donor DNA, PCR was used to amplify the QRDR region from two of the four transformed recipients and the nucleotide sequence was determined. In both transformants examined, the recipient cells harbored the same mutation carried by the donor DNA. In contrast, resistant cells arising after transformation with DNA amplified from a susceptible strain had a mutation different from that in the resistant parent strain, indicating that resistance in this case was due to spontaneous mutation or to errors incorporated in a small percentage of the PCR amplification product. These results demonstrate that the mutations in the PCR fragment could account for resistance to ciprofloxacin.

Attempts to express *H. pylori gyrA* in *E. coli* and complement *E. coli gyrA* mutants. To examine the ability of p19-10 and p19-4 to complement *gyrA* mutations, the plasmids were transformed into a temperature-sensitive *gyrA* mutant, *E. coli* KNK453 (13). Plasmids p19-10 and p19-4 were not able to restore growth at 43°C in *E. coli* KNK453, indicating either that the *H. pylori* gyrase was not expressed or that the *H. pylori gyrA* protein was not able to complement the *E. coli gyrA* protein. In contrast, control transformations using cloned *E. coli gyrA* (pSLS447 [30]) allowed strain KNK453 to grow at both 30°C and 43°C. To determine whether the *H. pylori gyrA* protein was being expressed, plasmids p19-10 and p19-4 were transformed into the minicell-producing *E. coli* SA2742 (14). As controls, pSLS447 and pUC19 were transformed into SA2742. Minicells were isolated, and expression products were labeled with [^{35}S]methionine and examined via SDS-gel electrophoresis and autoradiography. While pSLS447 produced a strong band in SDS gels running at 93 kDa corresponding to the *E. coli gyrA* peptide, none of the *H. pylori gyrA* clones produced expression products other than the plasmid-encoded β-lactamase (data not shown). These results indicate that the *H. pylori gyrA* was not expressed in the SA2742 host. In addition, the *H. pylori gyrA* gene was not expressed from p19-4 in a T7 RNA polymerase expression system (31).

DISCUSSION

Using ciprofloxacin alone to treat patients with *H. pylori* infections results in poor clearance of the organism and rapid conversion to ciprofloxacin resistance (3). We examined the *gyrA* gene from *H. pylori* to determine if quinolone resistance in this organism is due to mutation in the gyrase gene or whether some other cause, such as membrane permeability, might account for resistance. In addition, we were interested in examining the similarities among *gyrA* genes from *H. pylori* and other bacteria.

Our findings demonstrate that resistance to ciprofloxacin in *H. pylori* is primarily due to alterations within a relatively small portion of the *H. pylori gyrA* gene. Similar results have been obtained for *E. coli* (36), *S. aureus* (9, 29), and *C. jejuni* (33). In *E. coli* and in *C. jejuni* a mutation resulting in a substitution at Ser-83 is the most frequently reported *gyrA* mutation (33, 36). In contrast, our study revealed that the most common mutation in *H. pylori* was at Asp-91 (Asp-87 in *E. coli*). Strain UC561 had two mutations, one of which (corresponding to amino acid 93 in *E. coli*) has not been reported before. The MIC of ciprofloxacin for strains with two mutations is not different from the MICs for strains such as UC151, UC357, and others which had single substitutions at amino acid 91, suggesting that the additional mutation in UC561 does not contribute in an additive manner to the resistance level. Only 1 of 12 ciprofloxacin-resistant mutants did not have a mutation within the QRDR, suggesting that the primary cause for resistance is an alteration in the gyrase enzyme rather than other potential mechanisms of resistance. *H. pylori* is naturally competent and can be transformed with chromosomal DNA to streptomycin and metronidazole resistance (24, 34). Natural transformation has also been used for transposon shuttle mutagenesis of the *H. pylori* flagellin gene (10). We were able to transform *H. pylori* to ciprofloxacin resistance using a 238-bp fragment amplified from ciprofloxacin-resistant strains, thus demonstrating that resistance was due to amino acid substitutions encoded by the fragment. We observed a number of resistant transformants in control experiments, although the frequency of these was at least 2 orders of magnitude lower than the frequency obtained when DNA amplified from resistant strains was used. Resistant transformants obtained from control experiments were probably due to errors in nucleotide incorporation during the PCR amplification step rather than spontaneous mutation, since controls for which water replaced DNA in transformation experiments did not yield any resistant colonies. Although there were only a few, PCR errors were readily selected on media containing ciprofloxacin. Thus, transformation with PCR-generated fragments may provide a means of introducing mutations in *H. pylori* without using electroporation or shuttle vectors. The transformation experiments also demonstrate that ciprofloxacin resistance in *H. pylori* can result from amino acid substitutions within a relatively small range of the *gyrA* gene. This is probably the strongest supporting evidence for the role of specific amino acid changes orig-

inally described for *E. coli* (36). Other supporting evidence has been based on studies involving the entire gyrase gene and not introduction of a short segment of gyrase DNA with the mutation confirmed in the transformed strain. It supports existing information which illustrates a common region in bacterial *gyrA* proteins which accounts for quinolone resistance (27).

Homology comparisons revealed a strong similarity between the *gyrA* gene of *C. jejuni* and *H. pylori* and thus support the close phylogenetic relationship of the two bacterial genera. This relationship is further shown by the high degree of homology among *H. pylori*, *C. jejuni*, and *Campylobacter coli* flagellins (15, 16, 25). We found that the *H. pylori gyrA* gene was unable to complement *E. coli* gyrase mutations, presumably because the gene was not expressed in *E. coli*. Although the DNA upstream of the ATG start codon contained sequences similar to *E. coli* promoter consensus sequences, it is possible that the differences in the *H. pylori* promoter would not allow transcription of the gene. Alternatively, the gene was transcribed but not translated. It is interesting that the complete *gyrA* gene of *C. jejuni*, the closest matching sequence to *H. pylori gyrA*, could not be cloned in *E. coli* (33). Further study will be necessary to understand why the *H. pylori gyrA* gene is not expressed in *E. coli.*

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