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Mutations in 23S rRNA Are Associated with Clarithromycin Resistance in *Helicobacter pylori*

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Twelve clarithromycin-resistant *Helicobacter pylori* isolates (100% of resistant isolates examined) from seven different patients each contained an A→G transition mutation within a conserved loop of 23S rRNA. A→G transition mutations at positions cognate with *Escherichia coli* 23S rRNA positions 2058 and 2059 were identified. Clarithromycin-susceptible *H. pylori* isolates from 14 different patients displayed no polymorphisms in a conserved loop within domain V of 23S rRNA. The study is the first to report mutations in *H. pylori* associated with resistance to an antimicrobial agent used in established peptic ulcer treatment regimens.

The National Institutes of Health Consensus Conference in 1994 recommended that all patients with peptic ulcer disease and documented *Helicobacter pylori* infection be treated with appropriate antibacterial therapy (19). Multiple studies have demonstrated that cure of *H. pylori* infection results in cure of peptic ulcer disease (9, 12, 24). Cure of *H. pylori* infection may reduce the risk of gastric adenocarcinoma and has been associated with regression of gastric mucosa-associated lymphoid tissue (MALT) lymphoma (1, 23, 28). The highest cure rates have been obtained with multiple antimicrobial treatment regimens that include an imidazole (i.e., metronidazole) or clarithromycin and that usually last 7 to 14 days (for reviews, see references 13, 15, and 25). Resistance to clarithromycin accounted for 71% of treatment failures (five of seven treatment failures) in one limited study (3). In a separate study, Logan et al. (14) achieved a 78% cure rate with clarithromycin-omeprazole dual therapy. Ninety-three percent of *H. pylori* strains were susceptible to clarithromycin prior to treatment, but 20% of patients who were treatment failures yielded posttreatment isolates resistant to clarithromycin. Recent data from western Europe indicate that greater than 60% of treatment failures with clarithromycin-based protocols are associated with the presence of clarithromycin-resistant *H. pylori* isolates after therapy (4, 29).

Previous biochemical data, in addition to mutational analyses of 23S rRNA genes from various organisms (for a review, see reference 27), motivated us to examine domain V of 23S rRNA genes of clarithromycin-resistant *H. pylori* isolates. Twenty-eight *H. pylori* isolates were cultured from gastric antral biopsy specimens obtained from 17 different patients participating in clinical trials sponsored by Abbott Laboratories. Patients with duodenal ulcer disease confirmed by endoscopic evaluation were examined at different localities in the United States. No more than two patients were from a given locality, and all isolates were collected in 1993 or 1994. Patients were treated either with omeprazole only (omeprazole at 40 mg per day for 14 days [days 1 to 14] and then 20 mg per day for an additional 14 days [days 15 to 28]), clarithromycin only (clarithromycin at 500 mg three times per day for 14 days [days 1 to 14]), or clarithromycin and omeprazole (clarithromycin at 500 mg three times per day and omeprazole at 40 mg per day for 14 days [days 1 to 14] plus omeprazole at 20 mg per day for an additional 14 days [days 15 to 28]).

H. pylori isolates were passaged routinely on brain heart infusion agar containing 7% fresh horse blood and were incubated at 37°C with 12% CO_2 and 98% humidity. Agar dilution studies were performed on Mueller-Hinton agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 5% horse blood (BBL Microbiology Systems, Cockeysville, Md.) by an agar dilution method as described by Hardy et al. (10). Plates containing serial dilutions of the antibiotic were inoculated with approximately 10^6 CFU per spot by using a multipoint replicating device (Steer's replicator; Craft Machines, Chester, Pa.). Incubation was at 37°C for 3 days under microaerophilic conditions generated by CampyPak Plus (BBL Microbiology Systems) in GasPak jars.

Prior to harvesting of the cells, *H. pylori* isolates were examined microscopically following Gram staining to confirm the expected gram-negative, helical morphology. Isolates were verified as *H. pylori* by biochemical tests positive for catalase, oxidase, and urease activities. Genomic DNA was isolated from lysed *H. pylori* cells following phenol-chloroform extraction and ethanol precipitation as described previously (7). To examine the conserved domain V by DNA sequencing, primers 18 (5′-AGTCGGGACCTAAGGCGAG-3′; complementary to cognate *Escherichia coli* 23S rRNA positions 1342 to 1360) (16) and 21 (5′-TTCCCGCTTAGATGCTTTCAG-3′; complementary to positions 2765 to 2745) (16) amplified an approximately 1.4-kb amplicon in each isolate. PCR amplicons were purified by membrane filtration with Microcon-100 tubes according to the instructions of the manufacturer (Amicon, Beverly, Mass.). Approximately 400 ng of each purified amplicon was added as template DNA in cycle DNA sequencing reactions. Five picomoles of the internal 23S rRNA oligonucleotide primer 19 (5′-GTAGCGAAATTCCTTGTCGG-3′; complementary to cognate *E. coli* positions 1930 to 1949) (16) was end-labeled with γ-^{33}P by T4 polynucleotide kinase and was used as the sequencing primer. The 23S rRNA amplicons were sequenced by dideoxy termination during linear amplification according to the instructions of the manufacturer (dou-

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TABLE 1. Agar dilution MICs of clarithromycin and mutation profiles of *H. pylori* isolates

Isolate[a]	Treatment status[b]	Clarithromycin MIC (μg/ml)[c]	23S rRNA domain V
B	Pre-Rx	0.06 (S)	Wild type
C	Pre-Rx	0.015 (S)	Wild type
D1	Pre-Rx	16 (R)	A2059G
D2	Post-Rx; O	16 (R)	A2059G
E	Pre-Rx	0.03 (S)	Wild type
F[d]	Post-Rx; C	8 (R)	A2059G
G1	Pre-Rx	0.03 (S)	Wild type
G3[d]	Post-Rx; C	16 (R)	A2059G
G5[d]	Post-Rx; C	16 (R)	A2059G
H	Pre-Rx	0.015 (S)	Wild type
I1	Pre-Rx	0.06 (S)	Wild type
I2	Post-Rx; C	32 (R)	A2059G
I3	Post-Rx; C	32 (R)	A2059G
I4	Post-Rx; C	0.03 (S)	Wild type
I5	Post-Rx; C	0.06 (S)	Wild type
J2	Post-Rx; C	2 (I)	A2059G
J3	Post-Rx; C	4 (R)	A2059G
J4	Post-Rx; C	4 (R)	A2059G
K	Pre-Rx	0.06 (S)	Wild type
L	Pre-Rx	0.015 (S)	Wild type
M	Pre-Rx	0.06 (S)	Wild type
N	Pre-Rx	0.03 (S)	Wild type
O1	Pre-Rx	0.03 (S)	Wild type
O2	Post-Rx; C+O	128 (R)	A2058G
P	Pre-Rx	0.03 (S)	Wild type
Q1	Pre-Rx	0.03 (S)	Wild type
Q2[d,e]	Post-Rx; C+O	32 (R)	A2058G
R	Post-Rx; C	0.015 (S)	Wild type
11638	NCTC type strain[f]		Wild type

[a] Letters indicate different patients. Numbers indicate serial isolates from individual patients.
[b] Rx, treatment; O, omeprazole; C, clarithromycin.
[c] Comparable azithromycin MICs were obtained for each isolate. S, susceptible (MIC, ≤1.0 μg/ml); I, intermediate (MIC, 2.0 μg/ml); R, resistant (MIC, ≥4.0 μg/ml).
[d] Unhealed or subsequent disease recurrence.
[e] Presumptive heterozygote.
[f] NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England.

ble-stranded DNA Cycle Sequencing System; GIBCO BRL, Gaithersburg, Md.). A 50-bp region (cognate with *E. coli* positions 2046 to 2095) within domain V of 23S rRNA was analyzed and was manually aligned between the different *H. pylori* isolates.

The susceptibilities of the *H. pylori* isolates to the macrolides clarithromycin or azithromycin were evaluated by agar dilution experiments. Of 28 *H. pylori* isolates from 17 patients, 16 susceptible isolates from 14 patients and 12 clarithromycin-resistant isolates from 7 patients were obtained (Table 1). All clarithromycin-resistant isolates demonstrated cross-resistance to azithromycin (data not shown). MICs of clarithromycin for resistant isolates were variable (Table 1) and included MICs of 2 μg/ml (intermediate resistance; $n = 1$), 4 μg/ml ($n = 2$), 8 μg/ml ($n = 1$), 16 μg/ml ($n = 4$), 32 μg/ml ($n = 3$), and 128 μg/ml ($n = 1$). *H. pylori* isolates obtained from four patients with a posttreatment resistant isolate were susceptible to clarithromycin prior to treatment. Serial *H. pylori* isolates from the same patient that were susceptible and later resistant to clarithromycin yielded similar DNA fingerprints (data not shown), suggesting that individual mutant isolates arose within a single clone and were selected with antimicrobial treatment. Patient I yielded resistant isolates 1 and 2 months after monotherapy with clarithromycin. Interestingly, susceptible isolates

23S ribosomal RNA alleles	clarithro-mycin	23S rRNA mutation
A G A C G G A A A G A C C C C G U G G	S	wt
A G A C G G G A A G A C C C C G U G G	R	A2058G
A G A C G G A G A G A C C C C G U G G	R	A2059G

FIG. 1. Multiple sequence alignment depicting 23S rRNA allele sequences within a region of domain V. RNA sequences of a portion of domain V of 23S rRNA show the three allele sequences found in different clinical *H. pylori* isolates. Clarithromycin susceptibility (S) or resistance (R), determined by agar dilution, is listed. In the rightmost column, the specific mutation is indicated. wt, wild type. Point mutations are highlighted by the boxed residues.

were obtained 4 and 7 months after treatment. Wild-type *H. pylori* may thrive relative to mutant clones in the absence of therapeutic pressure in vivo. For example, *E. coli* clones containing a mutant 23S rRNA gene in a multicopy plasmid had extended doubling times in the absence of macrolides (26).

Cycle DNA sequencing of the 23S rRNA gene amplicons revealed the presence of point mutations in domain V associated with clarithromycin resistance. Two different A→G transition mutations at positions cognate with *E. coli* 23S rRNA positions 2058 and 2059 were identified in all resistant isolates (Fig. 1). Of the 12 mutations identified, 10 were A to G transitions at position 2059 (A2059G) (five patients) and 2 were A2058G (two patients). Three of seven patients with *H. pylori* strains containing mutations in 23S rRNA domain V either did not heal following treatment or had a recurrence of duodenal ulcer disease during the 6 to 7 months of follow-up evaluation (Table 1). The predominant transition mutation, A2059G, created an additional *Bsa*I site that was evident following *Bsa*I-mediated restriction digestion of 23S rRNA gene amplification products (Fig. 2). Isolates with the A2058G mutation did not create an additional *Bsa*I recognition site and appeared to be the wild type by *Bsa*I-mediated digestion (Fig. 2). None of the 16 susceptible isolates contained polymorphisms in the conserved loop region depicted in Fig. 1. One isolate, isolate Q2, demonstrated evidence of heterozygosity in the 23S rRNA gene (data not shown). Purification of a single colony of isolate Q2 and subsequent DNA sequencing yielded both A and G residues at position 2058. Since the population of bacteria in a single colony is clonal, presumably one copy of the 23S rRNA gene in isolate Q2 is that of the wild type and the other copy is that of the mutant.

Macrolides (22) such as erythromycin and clarithromycin inhibit nascent peptide chain elongation by interacting with the 50S ribosomal subunit and stimulating the release of peptidyl-tRNA from the A site (17). Biochemical studies have demonstrated a direct interaction of clarithromycin and its chief metabolite, 14-hydroxyclarithromycin, with 50S ribosomal subunits isolated from *H. pylori* (8). Footprinting experiments have demonstrated a direct physical interaction between erythromycin and the conserved region V of *E. coli* 23S rRNA (18). Specifically, the 23S rRNA residues A2058 and A2059 were protected when intact 70S ribosomes were incubated directly with erythromycin. Mutations conferring macrolide resistance occurred at or adjacent to the site of methylation (cognate to *E. coli* 23S rRNA position 2058) of 23S rRNA in *Bacillus stearothermophilus* (20), previously known to confer erythromycin (macrolide) resistance. The A2058G mutation has been associated with macrolide resistance in *E. coli* isolates containing the 23S rRNA gene in a multicopy plasmid (26), *Mycobacterium avium-M. intracellulare* (16), plant choroplasts (5, 11), and yeast mitochondria (21). Importantly, the principal muta-

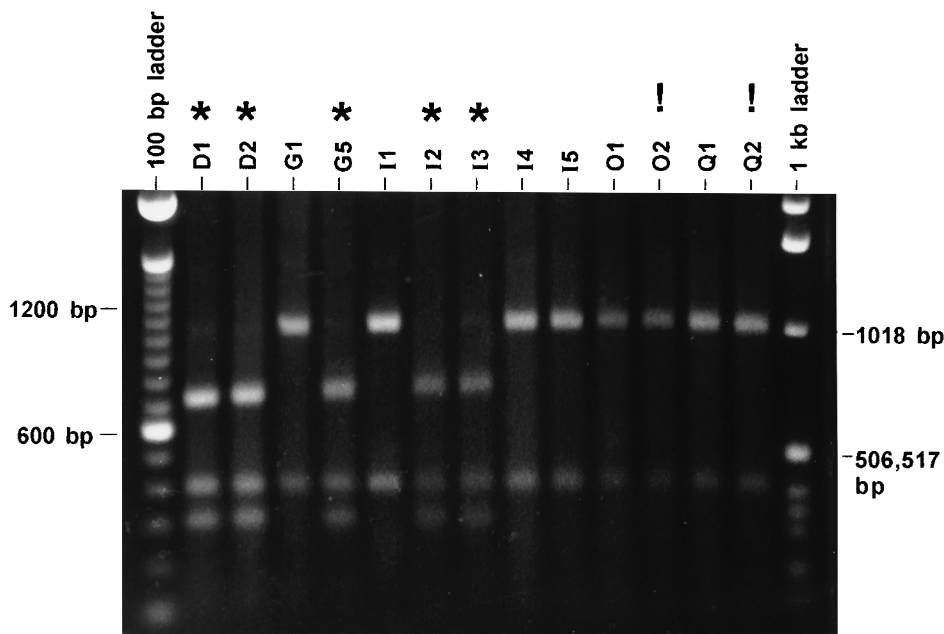

FIG. 2. Confirmation of A2059G mutations by *Bsa*I-mediated restriction digestion. A subset of *H. pylori* isolates is shown. Mutant isolates with the A2059G substitution are indicated by asterisks. Resistant isolates that contain the A2058G mutation and that show a wild-type profile are marked by exclamation points. Five-microliter aliquots containing amplicons generated with primers 18 and 21 (approximately 1.4 kb) were digested with 5 U of *Bsa*I at 55°C for 14 h. The presence of the A2059G mutation created an additional *Bsa*I restriction site [wild-type $(N)_5$AAGACC→mutant $(N)_5$GAGACC], causing the disappearance of the 1-kb fragment found after digestion of wild-type DNA. Two smaller products of approximately 300 and 730 bp appeared following the digestion of amplicons containing the A2059G mutation. Digested DNA was resolved in a 1% agarose gel containing 1× Tris-acetate-EDTA (TAE), and the gel was stained with ethidium bromide (0.5 μg/ml). DNA size markers (Gibco BRL) included a 100-bp ladder and a 1-kb ladder.

tion found in the present study, A2059G, has not been reported previously in bacteria, although it has been associated with lincomycin resistance in plant chloroplasts (5).

The current study presents evidence of the dominance of the mutant 23S rRNA allele. In contrast to *M. avium-M. intracellulare*, which possesses a single copy of the rRNA operon, two copies of the rRNA operon apparently exist in the *H. pylori* genome (2). One cannot exclude the possibility of variant 23S rRNA gene copy numbers in different *H. pylori* strains. Nevertheless, the heterozygosity of 23S rRNA or the presence of one mutant 23S rRNA gene and one wild-type 23S rRNA gene on the same *H. pylori* chromosome appears to confer a phenotype of clarithromycin resistance. A more complex heterozygous state in *E. coli* isolates with multiple wild-type 23S rRNA chromosomal genes and multicopy plasmids containing a mutant 23S rRNA gene was associated with macrolide resistance (6, 26). A single point mutation within domain V of 23S rRNA may be sufficient for conferring macrolide resistance in organisms containing a single rRNA operon, such as *Mycobacterium* spp. (16), or two rRNA operons, such as *H. pylori*. Although the use of macrolides such as erythromycin and clarithromycin has been widespread, the incidence of resistance in North American *H. pylori* isolates remains relatively low. Nevertheless, widespread oral therapy with macrolides for presumptive respiratory or gastrointestinal tract infections may increase the prevalence of resistant *H. pylori* isolates.

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