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Tetracycline-Resistant Clinical *Helicobacter pylori* Isolates with and without Mutations in 16S rRNA-Encoding Genes

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Tetracycline-resistant *Helicobacter pylori* strains have been increasingly reported worldwide. However, only a small number of tetracycline-resistant strains have been studied with regard to possible mechanisms of resistance and those studies have focused on mutations in the tetracycline binding sites of 16S rRNA-encoding genes. We here report studies of 41 tetracycline-resistant *H. pylori* strains (tetracycline MICs, 4 to 32 μg/ml) from North America ($n = 12$) and from East Asia ($n = 29$). DNA sequence analyses of 16S rRNA-encoding genes revealed that 22 (54%) of the resistant isolates carried one of five different single-nucleotide substitutions (CGA, GGA, TGA, AGC, or AGT) at the putative tetracycline binding site ($AGA_{965-967}$). Single-nucleotide substitutions were associated with reduced ribosomal binding and with slightly increased tetracycline MICs (1 to 2 μg/ml). The 19 tetracycline-resistant isolates with no detectable mutations in the tetracycline binding site had normal tetracycline-ribosome binding. All tetracycline-resistant isolates, including those with and those without mutations in the tetracycline binding site, showed decreased accumulation of tetracycline. These results suggest that tetracycline resistance is multifactorial, involving alterations both in ribosomal binding and in membrane permeability.

Helicobacter pylori infection is etiologically associated with chronic gastritis, peptic ulcers, gastric adenocarcinoma, and primary gastric lymphoma (4, 10). The antibiotics commonly used to treat *H. pylori* infection include amoxicillin, clarithromycin, tetracycline, and metronidazole given as combination therapy with two or more antibiotics plus an antisecretory drug and/or bismuth. Over time, the success rates of eradication therapy have fallen as the prevalence of antibiotic resistant *H. pylori* has increased (11, 12).

Tetracycline is a major component of quadruple therapy for *H. pylori* infection, and resistant *H. pylori* strains are presently uncommon in the United States and Europe (1, 21). However, tetracycline resistance has been increasingly reported in Brazil (22), Korea (14), Japan (15), Lebanon (31), Estonia (20), and India (32). A remarkable 59% of *H. pylori* strains from China were reported as resistant to tetracycline (37).

Tetracycline is a protein synthesis inhibitor, active against gram-positive and -negative bacteria, chlamydiae, mycoplasmas, rickettsia, and some protozoan parasites (2). Tetracycline inhibits bacterial growth by disrupting codon-anticodon interactions at the ribosome, specifically, by binding to the subunit of 30S, preventing attachment of aminoacyl-tRNA to the acceptor site. Photoaffinity labeling and chemical footprinting studies have shown that ribosomal protein S7 and 16S rRNA bases G693, A892, U1052, C1054, G1300, and G1338 all contribute to tetracycline binding (3, 24, 26, 30).

In *H. pylori* studies, tetracycline resistance has been attributed to mutations in the 16S rRNA-encoding genes that affect the binding site of tetracycline (8, 35). For example, studies of an isolate from The Netherlands (8) and of two isolates from Australia (35) showed that high-level tetracycline resistance (MIC, 8 to 64 μg/ml) was associated with triple mutations ($AGA_{965-967}$ to TTC) at the putative tetracycline binding site of the 16S rRNA-encoding genes. Six other tetracycline-resistant isolates with lower-level tetracycline resistance (MIC ≤ 4 μg/ml) also had single- or double-nucleotide substitutions at $AGA_{965-967}$ of the 16S rRNA-encoding genes (5). Here, we report characterization of tetracycline-resistant clinical *H. pylori* isolates from North America (United States and Canada) and East Asia (Korea and Japan), only approximately half of which had alterations in the putative tetracycline binding sites of 16S rRNA-encoding genes.

MATERIALS AND METHODS

***H. pylori* and culture conditions.** Tetracycline-resistant *H. pylori* strains were identified from 413 isolates obtained from the antrum and corpus of 227 patients from North America (United States, $n = 174$; Canada, $n = 53$). In addition, 29 tetracycline-resistant *H. pylori* strains from East Asia (Korea, $n = 22$; Japan, $n = 7$) that have been previously reported (15) were also included in this study. All stock cultures were maintained at $-80°C$ in brain heart infusion (BHI) broth supplemented with 20% glycerol (Sigma Co. St. Louis, Mo.). The frozen cultures were cultured on nonselective BHI agar plates when needed.

Determination of MIC. Drug MIC measurements for the *H. pylori* strains were performed by the serial twofold agar dilution method as described previously (16). Briefly, agar dilution plates were prepared using Mueller-Hinton agar as the base medium. Aged sheep blood (2 weeks old) was added to the Mueller-Hinton base medium at a concentration of 5%. The ranges for the antibiotic dilutions were 0.015 to 256 μg for amoxicillin, metronidazole, tetracycline (Sigma), and clarithromycin (Abbott Laboratories, Abbott Park, Ill.). Fresh *H. pylori* isolates (2- to 3-day cultures) were prepared in sterile saline and adjusted to a no. 2 McFarland standard. Using a Steers-type replicating device, 1 to 5 μl of the adjusted inoculum was delivered to the agar plates. All plates were

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incubated with CampyPak Plus (Becton Dickinson BBL, Cockeysville, Md.) at 37°C for 3 days. Metronidazole-resistant *H. pylori* ATCC 43504 was used as a quality control organism. Any test in which the drug MIC for the quality control organism was outside the approved range (64 to 256 μg of metronidazole/ml) was discarded, and the test was repeated. The resistance breakpoint used for tetracycline was a MIC of >2 μg/ml, as we proposed previously (14).

Genomic DNA extraction and random amplified polymorphism DNA (RAPD) analysis. *H. pylori* cells grown on BHI agar plates (2 plates) for 2 to 3 days were washed with TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA) and centrifuged at 15,000 rpm (12,000 × g) for 2 min. The cells were then suspended in lysis buffer (10 mM NaCl, 20 mM Tris HCl [pH 8.0], 25 mM EDTA, 0.5% sodium dodecyl sulfate, 100 μg of proteinase K/ml) and incubated at 50°C for 3 h. Cell lysates were extracted with phenol and chloroform (1:1). Genomic DNA was precipitated by ethanol and quantified by spectrophotometer at an optical density of 260 nm. The quality of the genomic DNA was assessed by 0.7% agarose gel electrophoresis with 1× TAE buffer (40 mM Tris HCl [pH 8.0], 20 mM sodium acetate, 1 mM EDTA) with ethidium bromide staining.

Each 100-μl reaction mixture of RAPD-PCR contained 50 ng of template DNA, 20 pmol of random amplified primer pMV 17B (17), 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl$_2$, 0.2 mM concentrations each of four deoxynucleoside triphosphates (Pharmacia LKB Biotechnology, Piscataway, N.J.), and 1 U of *Taq* polymerase (Promega, Madison, Wis.). PCR amplifications were performed with an automated thermocycler (MJ Research, New York, N.Y.) with an initial denaturation step (95°C for 5 min) followed by 40 cycles of denaturation (94°C for 30 s), annealing (37°C for 1 min), and extension (72°C for 1 min), with one final extension (72°C for 10 min). Aliquots (5 μl) of the PCR-amplified product were resolved in 1% agarose gels containing 0.5× TAE and stained with ethidium bromide. The RAPD-PCR amplifications were performed twice to confirm reproducibility of the genomic DNA fingerprinting for each *H. pylori* isolate. The DNA band patterns were visualized by ethidium bromide staining under a short-wavelength UV light source and photographed with Polaroid 667 film.

DNA sequence analysis of 16S rRNA-encoding genes. A portion of a 16S rRNA-encoding gene containing three putative tetracycline binding sites (8, 36) was amplified from the tetracycline-resistant *H. pylori* isolate. A 320-bp PCR fragment containing the putative tetracycline binding sites A (GGTGC$_{1052-1056}$) and C (AGA$_{965-967}$) was amplified by primer pair 16STCR-1 (5′-GGTAGTCC ACGCCCTAAACGA-3′) and 16STCR-2 (5′-GGGTTGCGCTCGTTGCGGG A-3′). A 464-bp PCR fragment containing the putative tetracycline binding site B (GACGT$_{1196-1200}$) was amplified by primer pair 16STCR-3 (5′-TCGTGTCG TGAGATGTTGGG-3′) and 16STCR-4 (5′-AGGAGGTGATCCAACCGCA G-3′). In addition, *rrnA*-specific primers (F1 and F2) and *rrnB*-specific primers (F3 and F4) used in combination with primer R-1 were also employed to determine data for both 16S rRNA-encoding genes as previously described (8). PCR conditions were identical to those previously described (16). Each PCR fragment was purified using a PCR product purification kit (QIAGEN Co., Hilden, Germany) before being used for DNA sequence determinations by use of a system from SeqWright (Houston, Tex.) or Macrogen (Seoul, Korea).

Natural transformation. Natural transformation was performed using the methods described by Haas et al. (13). Briefly, recipient *H. pylori* cells (10^7 per ml) were suspended in 10% horse serum BHI broth in a 50-ml Falcon tube and incubated for 6 h. The cells (200 μl) were dispensed into a 96-well microtiter plate with the addition of 1 μg of genomic DNA and incubated for 18 h under microaerobic conditions. Then, the cells were spread on selective BHI agar plates containing 0.5 to 2 μg of tetracycline per ml and incubated for up to 5 days.

Interruption of a *tetA* (P) gene in tetracycline-resistant *H. pylori*. To interrupt the *tetA* (P) gene (HP 1165) (33) from *H. pylori* ATCC 700392, a PCR fragment for the *tetA* (P) gene (1,161 bp of full length) was amplified by using PCR with a primer pair TetAB [5′-ATGTTAAGGAAAAACATTTTA-3′] and TetBE [5′-TCACTCATCAAACGGCTTA-3′] that covered from 1229436 to 1230597 of the *H. pylori* 26695 complete genome (33) and PCR conditions described previously (17). The PCR-amplified fragment was inserted into pBluescript SK+ (Stratagene, La Jolla, Calif.), and the fragment was cleaved by a restriction enzyme, SphI, which divided the *tetA* (P) gene into 320- and 841-bp lengths. A chloramphenicol resistance gene cassette (*cat*) (16) was inserted into the SphI site after blunt ends were made using a Klenow enzyme. The resulting recombinant plasmid containing *tetA* (P)::*cat* was used to transform tetracycline-resistant clinical *H. pylori* isolates 5255A and KH179A and the transformed resistant *H. pylori* cells as previously described (16).

Tetracycline-ribosome binding assay. Ribosomes from tetracycline-resistant clinical *H. pylori* isolates were purified using differential centrifugation as described by Doucet-Populaire et al. (6) and Goldman et al. (9) with modifications. Briefly, *H. pylori* cells grown on BHI agar plates (three plates) were harvested in

TABLE 1. MICs of tetracycline-resistant *H. pylori*

H. pylori isolate	MIC (μg/ml)[a]			
	Amoxicillin	Clarithromycin	Metronidazole	Tetracycline
ATCC 700392	0.125	0.031	2	0.25
(0203A)[b]	0.25	0.062	64	8
(1208A and -C)	0.25	0.062	8	4
2901A and -C	0.25	16	128	8
4442A	0.5	0.062	16	8
(4516C)	0.25	0.061	128	4
4923A and -C	0.25	0.061	8	4
5220A and -C	0.25	0.061	8	8
5255A	1	0.062	64	32
Transformed *H. pylori* colonies 700392 (5255A)[c]	0.25–0.5	0.062	4–8	4–8

[a] MIC measurements were repeated three times with identical results.
[b] Data in parentheses represent *H. pylori* isolates from Canada. A, *H. pylori* isolate from antrum; C, *H. pylori* isolate from corpus.
[c] 700392 (5255A) denotes colonies of *H. pylori* ATCC 700392 transformed using genomic DNA from clinical *H. pylori* 5255A. Transformed colonies (5 colonies) were used for three repeated MIC measurements with identical results.

phosphate buffer containing 137 mM NaCl and 2.7 mM KCl. The harvested cells were washed twice by centrifugation at 5,000 × g for 5 min at 4°C. The cells were resuspended in 4 ml of buffer A (10 mM Tris-HCl containing 4 mM MgCl$_2$, 100 mM KCl, and 10 mM NH$_4$Cl, pH 7.2) and passed through a French pressure cell (Aminco, Urbana, Ill.) at 600 lb/in^2. The cell debris was discarded by centrifugation at 30,000 × g for 30 min at 4°C. The supernatant was centrifuged at 100,000 × g for 90 min at 4°C to pellet the ribosomes. Ribosomes were resuspended in buffer A and stored at −80°C until needed.

The tetracycline-ribosome binding assay commenced by the addition of 75 pmol of [7-^{3}H]tetracycline to different amounts of ribosomes (at an optical density at 260 nm of 1, 2, or 3) in 0.5 ml of buffer A as a reaction mixture. After 30 min, the binding reaction was stopped by diluting the reaction mixture with 3 ml of cold buffer B (10 mM Tris hydrochloride containing 5 mM MgCl$_2$ and 150 mM KCl). The ribosomes were collected on a 0.45-μm-pore-size nitrocellulose filter (Millipore). After three washes with buffer B (3 ml), the filters were transferred to scintillation vials containing scintillation fluid (CytoScint; Fisher Biotech) for radioactivity determination in a Beckman LS 6500 scintillation counter (Beckman Instruments, Palo Alto, Calif.).

Antibiotic accumulation assay. Antibiotic accumulation in *H. pylori* cells was performed as previously described (18). Briefly, *H. pylori* cells were grown on BHI agar plates without any antibiotic for 2 to 3 days and harvested in 10 ml of assay buffer containing 50 mM KPO$_4$ and 1 mM MgSO$_4$ (pH 6.6). The cells (5 × 10^9 per ml) were centrifuged and resuspended in 10 ml of the same assay buffer. Antibiotic accumulation assays commenced by the addition of [7-^{3}H]tetracycline (Dupont/NEN Research Product, Boston, Mass.) (0.6 Ci mmol$^-$; 22.2 GBq mmol$^-$). Aliquots (1 ml) were taken every 10 min. After 20 min, each cell suspension was divided in half and 100 μM of CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) was added to only one half to de-energize the cells but not the other half. Then, 1 ml aliquots were taken every 10 min from each half. Each aliquot was then immediately centrifuged and washed three times in phosphate-buffered saline. The resulting pellets were then diluted in scintillation fluid (CytoScint; Fisher Biotech) and analyzed for radioactivity in a Beckman LS 6500 scintillation counter (Beckman Instruments).

RESULTS

Tetracycline-resistant *H. pylori*. Twelve tetracycline-resistant *H. pylori* isolates (tetracycline MICs, 4 to 32 μg/ml) were taken from 8 (4%) of the 227 North American patients. MICs of amoxicillin, clarithromycin, metronidazole, and tetracycline were measured for the 12 tetracycline-resistant isolates and compared to the susceptible reference *H. pylori* ATCC 700392 results (Table 1). In addition, we studied 29 isolates from East Asia that were reported previously (15). Tetracycline resistance levels among these isolates resulted in tetracycline MICs that ranged from 4 to 16 μg/ml (15).

TABLE 2. Nucleotide sequence analysis of putative tetracycline binding sites of 16S rRNA-encoding genes from tetracycline-resistant *H. pylori* isolates

Type of nucleotide substitution at site C ($AGA_{965-967}$)[a]	Tetracycline resistant *H. pylori* isolate(s)[b]	
	North American isolate(s)[d]	East Asian isolate(s)[c]
gGA	(0203A), 2901C, (4516C), 4923A, 4923C	KH84B, KH87B, KH95B, KH164B, KH179A, KH185A, KH259A, KH330A
AGc	(1208A), (1208C)	KH69A, KH400A
AGt	2901A	
cGA		KH100A
tGA	5220A, 5220C	KH430B
No nucleotide change	4442A, 5255A	KH55A, KH161A, KH222A, KH292B, KH294A, KH299A, KH422B, KH439A, KH453A, KH461A, JH34A, JH86A, JH86B, JH98A, JH223A, JH244B, JH269B

[a] Altered nucleotides were shown as a lowercase.
[b] A, *H. pylori* isolates from antrum; C or B, *H. pylori* isolates from corpus or body.
[c] East Asian isolates data were from a previous report (15).
[d] Data in parentheses represent *H. pylori* isolates from Canada.

Genotype analysis. Tetracycline-resistant isolates from the eight North American patients were used for RAPD-PCR fingerprinting genotype analyses. Each isolate had a unique fingerprint. In contrast, fingerprints of paired antrum-corpus isolates were identical except for those for one patient (patient 2901), who was apparently infected by two different tetracycline-resistant *H. pylori* strains.

DNA sequence analysis of 16S rRNA-encoding genes from tetracycline-resistant *H. pylori* cells. Three putative tetracycline binding sites of 16S rRNA-encoding genes (16S *rrnAB*) were examined by DNA sequence determination of the 41 tetracycline-resistant clinical isolates. DNA sequence alterations for the North American isolates were found only in the putative tetracycline binding site C ($AGA_{965-967}$) of 16S *rrnAB* and consisted of single-nucleotide substitutions (A_{965} to G or T; A_{967} to C or T) (Table 2). The nucleotide substitutions in paired antrum-corpus isolates (patients 1208, 4923, and 5220) were identical with the exception of the nucleotide substitutions in site C from isolates 2901A and 2901C, which were different (i.e., 2901A, A_{967} to T; 2901C, A_{965} to G). Two resistant isolates (4442A and 5255A) did not show nucleotide substitutions in any of the three putative tetracycline binding sites (Table 2).

DNA sequence alterations for the East Asia were also found only in putative tetracycline binding site C ($AGA_{965-967}$) of 16S *rrnAB* and consisted of single-nucleotide substitutions (A_{965} to G, C, or T; A_{967} to C). However, 17 isolates had no detectable mutations in any of putative tetracycline binding sites of 16S *rrnAB*. Overall, five different types of single-nucleotide substitutions ($AGA_{965-967}$ to GGA, AGC, AGT, CGA, and TGA) were identified from 22 tetracycline-resistant isolates, and no mutation in any of the putative tetracycline binding sites of 16S *rrnAB* was found in the other isolates (19 of 41; 46%). The most common alteration was a single-nucleotide substitution of $AGA_{965-967}$ to GGA at putative tetracycline binding site C, which was present in both North American and East Asian isolates (Table 2).

Role of single-nucleotide substitutions of the 16S rRNA-encoding genes in tetracycline resistance. Each of the five different single-nucleotide substitutions was assessed regarding its potential role in tetracycline resistance. PCR-amplified DNA fragments carrying the different nucleotide substitutions were used to transform susceptible *H. pylori* ATCC 700392. Transformants were selected on agar plates containing only 0.5 to 1 μg of tetracycline per ml. The same nucleotide substitutions were found in the tetracycline-resistant transformants. For example, nucleotide substitution of $AGA_{965-967}$ to GGA in the PCR fragment was always found in the tetracycline-resistant transformants used for the same PCR fragment. These results confirm that the single-nucleotide substitution at site C appears to be involved in low-level resistance to tetracycline, as reported previously (7).

To further analyze the role of single-nucleotide substitutions, tetracycline-ribosome binding assays were performed using the clinical isolates carrying nucleotide substitutions in ribosome binding sites. Ribosomes were isolated from the tetracycline-resistant clinical isolates carrying the different types of nucleotide substitutions (isolates 1208A, 2901A, 4923A, and 5220A), a resistant isolate without a nucleotide substitution in 16S rRNA-encoding genes (isolate 5255A), and the susceptible *H. pylori* ATCC 700392 as a control. Tetracycline binding to the ribosomes with nucleotide substitutions was decreased 24 to 52% compared to the susceptible *H. pylori* results (Fig. 1). However, tetracycline binding to the ribosomes from the tetracycline-resistant isolate, 5255A, which had no nucleotide substitution in 16S rRNA-encoding genes, was similar to that of the susceptible *H. pylori* (Fig. 1).

Role of putative tetracycline resistance protein [*tetA* (P)] in tetracycline resistance. The putative tetracycline resistance protein [*tetA* (P)] was identified from the complete *H. pylori* genome (strain 2669) (33). However, the *tetA* (P) gene may not be functional for production of tetracycline resistance, because strain 26695 is susceptible to tetracycline. To test whether the *tetA* (P) gene was involved in tetracycline resistance among the tetracycline-resistant clinical isolates, we interrupted the *tetA* (P) gene in resistant clinical isolates 5255A and KH179A and in the transformed resistant strains by use of a chloramphenicol resistance gene cassette as described in Materials and Methods. All of the *tetA* (P)-interrupted resistant strains remained resistant to tetracycline at levels identical to those seen with the parental resistant strains, suggesting that the *tetA* (P) gene was not be involved in the tetracycline resistance in these strains.
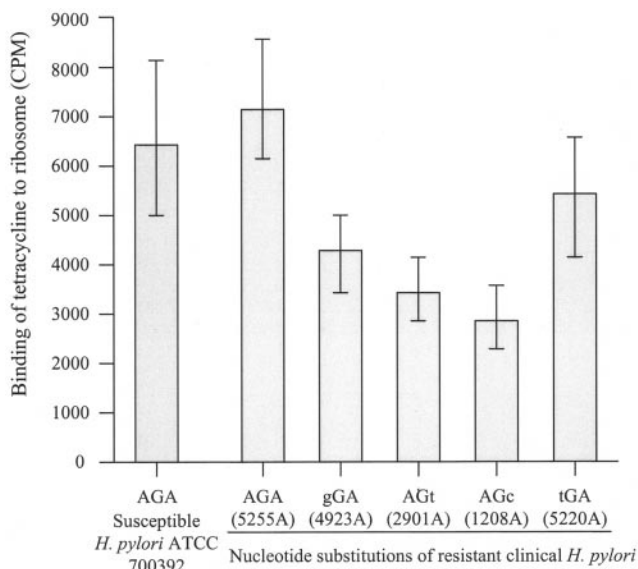
FIG. 1. Patterns of ribosome binding to tetracycline in tetracycline-susceptible and -resistant *H. pylori*. Tetracycline-resistant *H. pylori* 5255A has no nucleotide substitution, and other tetracycline-resistant *H. pylori* isolates have nucleotide substitutions in the 16S rRNA-encoding genes. *H. pylori* ATCC 700392, a tetracycline-susceptible strain, was used as a positive control. The results represent the means of three independent experiments; the vertical bars represent standard deviations.

The role of genomic DNA extracted from tetracycline-resistant *H. pylori* in tetracycline resistance. Genomic DNA extracted from the tetracycline-resistant clinical isolate without nucleotide substitutions among the three putative tetracycline binding sites of the 16S rRNA-encoding genes (isolate 5255A) was used for natural transformation of susceptible *H. pylori* ATCC 700392. Several transformed *H. pylori* colonies grew on BHI agar plates containing 2 μg of tetracycline per ml after 3 to 4 days of incubation whereas there was no growth of the negative control (i.e., without a DNA source) on the same agar plates. DNA sequence analyses of five transformed tetracycline-resistant *H. pylori* colonies confirmed the absence of nu-

cleotide substitutions in the 16S rRNA-encoding genes. Tetracycline MICs for the five transformed resistant *H. pylori* colonies ranged from 4 to 8 μg/ml (Table 1).

The role of genomic DNA extracted from the tetracycline-resistant clinical isolate with a nucleotide substitution among the tetracycline binding sites of the 16S rRNA-encoding genes was also examined. These experiments used the transformants reported previously (15), which were derived from susceptible *H. pylori* strains ATCC 700392 and ATCC 43629 by use of genomic DNA from the clinical resistant *H. pylori* strains KH84B and KH179A. DNA sequences of the 16S rRNA-encoding genes were examined among three transformants from the two susceptible recipient *H. pylori* strains (a total 12 transformants: 3 from *H. pylori* ATCC 700392/KH84B, 3 from *H. pylori* ATCC 700392/KH179A, 3 from *H. pylori* ATCC 43629/KH84B, and 3 from *H. pylori* ATCC 43629/KH179A). All transformants, except ATCC 700392/KH84B and ATCC 700392/KH179A, contained the nucleotide substitution at putative tetracycline binding site C (AGA$_{965-967}$ to GGA). The transformants ATCC 700392/KH84B and ATCC 700392/KH179A, however, did not have detectable mutations at any of the putative tetracycline binding sites. These results suggest that although a single-nucleotide substitution in the 16S rRNA-encoding genes is associated with low levels of tetracycline resistance, additional determinants transferred from the donor genomic DNA to the susceptible *H. pylori* strains are needed to produce tetracycline MICs in the range of 4 to 8 μg/ml.

Role of tetracycline accumulation in resistance. To further elucidate possible tetracycline resistance mechanisms, we examined tetracycline accumulation and active efflux of tetracycline as previously described (18). We used the clinical resistant *H. pylori* 5255A and the transformed resistant *H. pylori* ATCC 700392 (5255A) isolates and also included the susceptible parental *H. pylori* ATCC 700392 strain as a control. Tetracycline accumulations were decreased in both the transformed resistant *H. pylori* and the clinical resistant *H. pylori* 5255A strains. However, the degree of the reduction in the level of the resistant transformant was less than that of the resistant clinical isolate (20 to 80% reduction of the resistant clinical isolate) (Fig. 2A). This change may reflect the fact that
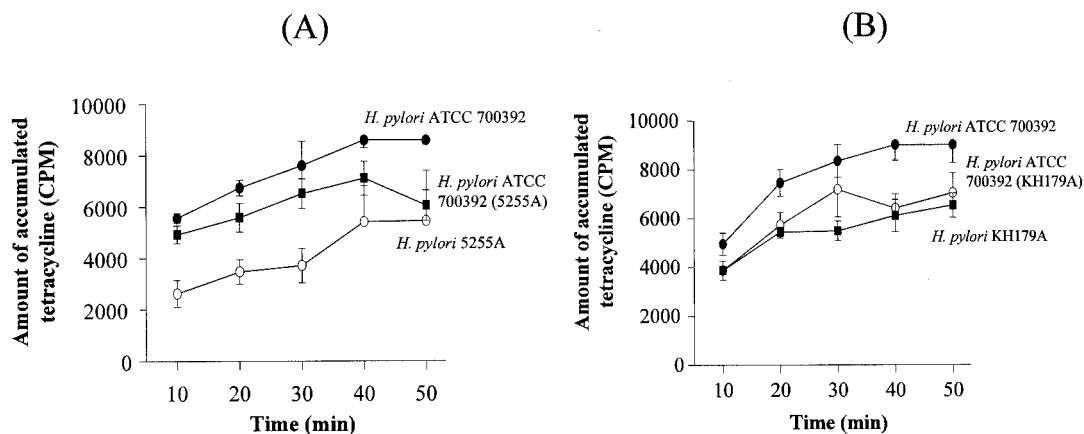
(A)　　　　　　　　　　　　　　　　(B)

FIG. 2. Tetracycline accumulation assays for susceptible *H. pylori* ATCC 700392, transformed tetracycline-resistant *H. pylori* ATCC 700392 (5255A) and *H. pylori* ATCC 700392 (KH179A), and tetracycline-resistant clinical *H. pylori* 5255A and *H. pylori* KH179A. The results represent the means of three independent experiments; the vertical bars represent standard deviations.

MIC levels for the transformants (tetracycline MIC, 4 to 8 μg/ml) were less than that for the clinical isolate (tetracycline MIC, 32 μg/ml) (Table 1). Decreased accumulation of tetracycline was also observed with the clinical resistant *H. pylori* KH179A isolate and the transformed resistant *H. pylori* ATCC 700392 (KH179A) isolate reported previously (15) (Fig. 2B). We also examined whether the resistant isolates were associated with an energy-coupled active efflux mechanism by using CCCP as a de-energizing agent. Addition of CCCP at 20 min in the accumulation assays did not increase tetracycline accumulation in any of the strains, and identical results were repeatedly obtained (data not shown). Overall, these observations suggest that the decreased accumulation of tetracycline may be one of important tetracycline resistance mechanisms responsible for the development of high-level tetracycline resistance.

DISCUSSION

Tetracycline-resistant *H. pylori* strains have increasingly been reported worldwide (1, 14, 22, 23, 28, 37). In this study, 4% of a large group of patients from North America had tetracycline-resistant *H. pylori* strain infections. RAPD-PCR fingerprinting showed that the resistant isolates from different patients were all of different genotypes, suggesting that the resistance likely does not reflect clonal dissemination of a specific resistant determinant. The tetracycline MICs for the tetracycline-resistant isolates initially ranged from 4 to 32 μg/ml. However, extended growth or step-wise increases in tetracycline concentrations resulted in an increased MIC level up to 64 μg/ml, suggesting that high-level tetracycline resistance results from the cumulative effects of multiple factors.

DNA sequence analyses of putative tetracycline binding sites of 16S rRNA-encoding genes among tetracycline-resistant isolates showed that 54% of tetracycline-resistant isolates contained a single-nucleotide substitution at tetracycline binding site C. Although we confirmed that it was possible to make transformants by use of PCR fragments carrying the single-nucleotide substitutions (7, 8, 35), transformation of susceptible *H. pylori* strains with single-nucleotide substitutions was associated with lower-level tetracycline MICs than those seen with the parental resistant strain (e.g., MICs of 1 or 2 μg/ml versus ≥4 μg/ml). We also extend prior observations by confirming that single-nucleotide substitutions (A_{965} to G or T substitutions and A_{967} to C or T substitutions) were associated with a reduction in tetracycline binding to ribosomes.

Importantly, approximately half (46%) of tetracycline-resistant clinical isolates did not contain nucleotide substitutions at any putative tetracycline binding sites of 16S rRNA-encoding genes. These isolates also expressed higher levels of MICs than those associated with single-nucleotide substitutions in binding site C, suggesting that additional determinants were responsible for the higher levels of resistance. Our data showing the presence of additional resistant determinants in the genomic DNA include the following. (i) Many tetracycline-resistant isolates had no nucleotide substitutions in any of the three putative tetracycline binding sites of the 16S rRNA-encoding genes. (ii) A tetracycline-resistant isolate without a nucleotide substitution at any of the three putative tetracycline binding sites showed tetracycline-ribosome binding similar to that seen with the tetracycline-susceptible *H. pylori* isolate. (iii) Genomic

DNA from the resistant isolate containing a single-nucleotide substitution in the 16S rRNA-encoding genes resulted in higher tetracycline MICs than that seen with the single-nucleotide substitutions. (iv) Genomic DNA from the resistant isolate without any nucleotide substitution at any of the three putative tetracycline binding sites could increase the tetracycline MIC by >2 μg/ml. (v) All transformed tetracycline-resistant *H. pylori* isolates with a single-nucleotide substitution exhibited lower MIC levels than those of clinical resistant isolates. (vi) Interruption of *tetA* (P) did not change the level of tetracycline resistance.

Several tetracycline resistance mechanisms have been reported in studies of bacteria (2). In most cases, tetracycline efflux pumps that are specific for the active transport of tetracycline are responsible for tetracycline resistance (19). A second mechanism of tetracycline resistance is mediated through ribosomal protection proteins that reduce the affinity of ribosomes for tetracycline or release the bound tetracycline from the ribosome (34). The third mechanism is an as-yet-unknown chemical modification of tetracycline by an oxidoreductase that requires NADP (2). The fourth mechanism of resistance is a mutation in the 16S rRNA-encoding genes that affects the binding site of tetracycline (7, 29, 35). Tetracycline resistance can be also acquired by decreased membrane permeability, and this is often coupled with additional resistance mechanisms (e.g., active drug efflux, enzymatic degradation of antibiotics, or target alterations) producing high levels of tetracycline resistance (18, 25, 27). We hypothesize that one tetracycline resistance mechanism in *H. pylori* is decreased membrane permeability and/or active efflux of tetracycline. This idea is based on the fact that the resistant isolates, including the resistant transformant, showed at least slightly increased MIC levels of the tested antibiotics. Our data clearly showed that the tetracycline-resistant clinical isolates had decreased membrane permeability but did not show clear evidence for energy-coupled active efflux when the de-energizing agent CCCP was used. However, these results do not completely exclude the possibility of the active efflux in the resistant strains, because we did not test other de-energizing agents such as arsenate or 2,4-dinitrophenol. Studies with the other de-energizing agents will be needed to confirm whether the tetracycline-resistant isolates are associated with energy-coupled active efflux.

The decreased membrane permeability associated with tetracycline resistance is similar to a result presented in our previous report, namely, that amoxicillin-resistant clinical *H. pylori* was also cross-resistant to tetracycline and that this effect was also mediated by decreased membrane permeability (18). However, total outer membrane protein profiles of the resistant clinical and transformed strains in this study (data not shown) were not similar to those seen with the previously investigated strains (18). It is not clear whether mechanisms of decreased accumulation reported for the previous study (18) and the present study are identical or whether the outer membrane protein profiles and MICs are different between the tetracycline-resistant isolates examined in the previous study and those examined in this study. In summary, our results suggest that tetracycline resistance among clinical *H. pylori* isolates may be associated with nucleotide substitutions in the tetracycline binding site C of the 16S rRNA-encoding genes as well as with decreased accumulation of tetracycline.

REFERENCES

1. **Boyanova, L., A. Mentis, M. Gubina, E. Rozynek, G. Gosciniak, S. Kalenic, V. Goral, L. Kupcinskas, B. Kantarceken, A. Aydin, A. Archimandritis, D. Dzierzanowska, A. Vcev, K. Ivanova, M. Marina, I. Mitov, P. Petrov, A. Ozden, and M. Popova.** 2000. The status of antimicrobial resistance of *Helicobacter pylori* in Eastern Europe. Clin. Microbiol. Infect. **8:**388–396.

2. **Chopra, I., and M. Roberts.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. **65:**232–260.

3. **Chopra, I., P. M. Hawkey, and M. Hinton.** 1992. Tetracyclines, molecular and clinical aspects. J. Antimicrob. Chemother. **29:**245–277.

4. **Crump, M., M. Gospodarowicz, and F. A. Shepherd.** 1999. Lymphoma of the gastrointestinal tract. Semin. Oncol. **26:**324–337.

5. **Dailidiene, D., M. T. Bertoli, J. Miciuleviciene, A. K. Mukhopadhyay, G. Dailide, M. A. Pascasio, L. Kupcinskas, and D. E. Berg.** 2002. Emergence of tetracycline resistance in *Helicobacter pylori*: multiple mutational changes in 16S ribosomal DNA and other genetic loci. Antimicrob. Agents Chemother. **46:**3940–3946.

6. **Doucet-Populaire, F., C. Truffot-Pernot, J. Grosset, and V. Jarlier.** 1995. Acquired resistance in *Mycobacterium avium* complex strains isolated from AIDS patients and beige mice during treatment with clarithromycin. J. Antimicrob. Chemother. **36:**129–136.

7. **Gerrits, M. M., M. Berning, A. H. van Vliet, E. J. Kuipers, and J. G. Kusters.** 2003. Effects of 16S rRNA gene mutations on tetracycline resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **47:**2984–2986.

8. **Gerrits, M. M., M. R. De Zoete, N. L. Arents, E. J. Kuipers, and J. G. Kusters.** 2002. 16S rRNA mutation-mediated tetracycline resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **46:**2996–3000.

9. **Goldman, R. C., D. Zakula, R. Flamm, J. Beyer, and J. Capobianco.** 1994. Tight binding of clarithromycin, its 14-(*R*)-hydroxy metabolite, and erythromycin to *Helicobacter pylori* ribosomes. Antimicrob. Agents Chemother. **38:**1496–1500.

10. **Graham, D. Y.** 1997. *Helicobacter pylori* infection in the pathogenesis of duodenal ulcer and gastric cancer: a model. Gastroenterology **113:**1983–1991.

11. **Graham, D. Y.** 1998. Antibiotic resistance in *Helicobacter pylori*: implications for therapy. Gastroenterology **115:**1272–1277.

12. **Graham, D. Y.** 2000. Therapy of *Helicobacter pylori*: current status and issues. Gastroenterology **118:**S2–S8.

13. **Haas, R., T. F. Meyer, and J. P. van Putten.** 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. Mol. Microbiol. **8:**753–760.

14. **Kim, J. J., R. Reddy, M. Lee, J. G. Kim, F. A. El-Zaatari, M. S. Osato, D. Y. Graham, and D. H. Kwon.** 2001. Analysis of metronidazole, clarithromycin and tetracycline resistance of *Helicobacter pylori* isolates from Korea. J. Antimicrob. Chemother. **47:**459–461.

15. **Kwon, D. H., J. J. Kim, M. Lee, Y. Yamaoka, M. Kato, M. S. Osato, F. A. El-Zaatari, and D. Y. Graham.** 2000. Isolation and characterization of tetracycline-resistant clinical isolates of *Helicobacter pylori*. Antimicrob. Agents Chemother. **44:**3203–3205.

16. **Kwon, D.-H., F. A. K. El-Zaatari, M. Kato, M. S. Osato, R. Reddy, Y. Yamaoka, and D. Y. Graham.** 2000. Analysis of *rdxA* and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of *Helicobacter pylori*. Antimicrob. Agents Chemother. **44:**2133–2142.

17. **Kwon, D. H., K. Hulten, M. Kato, J. J. Kim, M. Lee, F. A. K. El-Zaatari, M. S. Osato, and D. Y. Graham.** 2001. DNA sequence analysis of *rdxA* and *frxA* from 12 pairs of metronidazole-sensitive and -resistant clinical *Helicobacter pylori* isolates. Antimicrob. Agents Chemother. **45:**2609–2615.

18. **Kwon, D. H., M. P. Dore, J. J. Kim, M. Kato, M. Lee, J. Y. Wu, and D. Y. Graham.** 2003. High-level β-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **47:**2169–2178.

19. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.

20. **Loivukene, K., H. I. Maaroos, H. Kolk, I. Kull, K. Labotkin, and M. Mikelsaar.** 2002. Prevalence of antibiotic resistance of *Helicobacter pylori* isolates in Estonia during 1995–2000 in comparison to the consumption of antibiotics used in treatment regimens. Clin. Microbiol. Infect. **8:**598–603.

21. **Mégraud, F.** 1997. Resistance of *Helicobacter pylori* to antibiotics. Aliment. Pharmacol. Ther. **11**(Suppl. 1):43–53.

22. **Mendonca, S., C. Ecclissato, M. S. Sartori, A. P. Godoy, R. A. Guerzoni, M. Degger, and J. Pedrazzoli, Jr.** 2000. Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxicillin, tetracycline, and furazolidone in Brazil. Helicobacter **5:**79–83.

23. **Midolo, P. D., M. G. Korman, J. D. Turnidge, and J. R. Lambert.** 1996. *Helicobacter pylori* resistance to tetracycline. Lancet **347:**1194–1195.

24. **Moazed, D., and H. F. Noller.** 1987. Interaction of antibiotics with functional sites in 16S ribosomal RNA. Nature **327:**389–394.

25. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–387.

26. **Oehler, R., N. Polacek, G. Steiner, and A. Barta.** 1997. Interaction of tetracycline with RNA: photoincorporation into ribosomal RNA of *Escherichia coli*. Nucleic Acids Res. **25:**1219–1224.

27. **Putman, M., H. W. van Veen, and W. N. Konings.** 2000. Molecular properties of bacterial multidrug transporters. Microb. Mol. Biol. Rev. **64:**672–693.

28. **Realdi, G., M. P. Dore, A. Piana, A. Atzei, M. Carta, L. Cugia, A. Manca, B. M. Are, G. Massarelli, I. Mura, A. Maida, and D. Y. Graham.** 1999. Pretreatment antibiotic resistance in *Helicobacter pylori* infection: results of three randomized controlled studies. Helicobacter **4:**106–112.

29. **Ross, J. I., E. A. Eady, J. H. Cove, and W. J. Cunliffe.** 1998. 16S rRNA mutation associated with tetracycline resistance in a gram-positive bacterium. Antimicrob. Agents Chemother. **42:**1702–1705.

30. **Schnappinger, D., and W. Hillen.** 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch. Microbiol. **165:**359–369.

31. **Sharara, A. I., M. Chedid, G. F. Araj, K. A. Barada, and F. H. Mourad.** 2002. Prevalence of *Helicobacter pylori* resistance to metronidazole, clarithromycin, amoxicillin and tetracycline in Lebanon. Int. J. Antimicrob. Agents **19:**155–158.

32. **Thyagarajan, S., P. Ray, B. K. Das, A. Ayyagari, A. A. Khan, S. Dharmalingam, U. A. Rao, P. Rajasambandam, B. Ramathilagam, M. Sharma, S. Naik, and C. Habibullah.** 2003. Geographical difference in antimicrobial resistance pattern of *Helicobacter pylori* clinical isolates from Indian patients: Multicentric study. J. Gastroenterol. Hepatol. **18:**1373–1378.

33. **Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenny, L. M. Fitzegerald, N. M. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hays, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539–547.

34. **Trieber, C. A., N. Burkhardt, K. H. Nierhaus, and D. E. Taylor.** 1998. Ribosomal protection from tetracycline mediated by Tet(O): Tet(O) interaction with ribosomes is GTP-dependent. Biol. Chem. **379:**847–855.

35. **Trieber, C. A., and D. E. Taylor.** 2002. Mutations in the 16S rRNA genes of *Helicobacter pylori* mediate resistance to tetracycline. J. Bacteriol. **184:**2131–2140.

36. **Wimberly, B. T., D. E. Brodersen, W. M. Clemons, Jr., R. J. Morgan-Warren, A. P. Carter, C. Vonrhein, T. Hartsch, and V. Ramakrishnan.** 2000. Structure of the 30S ribosomal subunit. Nature **407:**327–339.

37. **Wu, H., X. D. Shi, H. T. Wang, and J. X. Liu.** 2000. Resistance of *Helicobacter pylori* to metronidazole, tetracycline and amoxycillin. J. Antimicrob. Chemother. **46:**121–123.