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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*

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Metronidazole (Mtz) is a critical ingredient of modern multidrug therapies for *Helicobacter pylori* infection. Mtz resistance reduces the effectiveness of these combinations. Although null mutations in a *rdxA* gene that encodes oxygen-insensitive NAD(P)H nitroreductase was reported in Mtz-resistant *H. pylori*, an intact *rdxA* gene has also been reported in Mtz-resistant *H. pylori*, suggesting that additional Mtz resistance mechanisms exist in *H. pylori*. We explored the nature of Mtz resistance among 544 clinical *H. pylori* isolates to clarify the role of *rdxA* inactivation in Mtz resistance and to identify another gene(s) responsible for Mtz resistance in *H. pylori*. Mtz resistance was present in 33% (181 of 544) of the clinical isolates. There was marked heterogeneity of resistance, with Mtz MICs ranging from 8 to ≥256 μg/ml. *rdxA* inactivation resulted in Mtz MICs of up to 32 μg/ml for 6 Mtz-sensitive *H. pylori* strains and 128 μg/ml for one Mtz-sensitive strain. Single or dual (with *rdxA*) inactivation of genes that encode ferredoxin-like protein (designated *fdxB*) and NAD(P)H flavin oxidoreductase (*frxA*) also increased the MICs of Mtz for sensitive and resistant strains with low to moderate levels of Mtz resistance. *fdxB* inactivation resulted in a lower level of resistance than that from *rdxA* inactivation, whereas *frxA* inactivation resulted in MICs similar to those seen with *rdxA* inactivation. Further evidence for involvement of the *frxA* gene in Mtz resistance included the finding of a naturally inactivated *frxA* but an intact *rdxA* in an Mtz-resistant strain, complementation of Mtz sensitivity from an Mtz-sensitive strain to an Mtz-resistant strain or vice versa by use of naturally inactivated or functional *frxA* genes, respectively, and transformation of an Mtz-resistant *Escherichia coli* strain to an Mtz sensitive strain by a naturally functional *frxA* gene but not an inactivated *frxA* gene. These results are consistent with the hypothesis that null mutations in *fdxB*, *frxA*, or *rdxA* may be involved in Mtz resistance.

Helicobacter pylori is recognized as the major cause of peptic ulcer disease and a risk factor for gastric adenocarcinoma and primary gastric lymphoma (4, 34, 35). *H. pylori* infection is one of the most common infections worldwide and accounts for tremendous morbidity and mortality. Clinical experience has demonstrated that *H. pylori* infection is not easy to cure. The primary impediments to successful treatment are lack of compliance with the drug regimens and development of antibiotic-resistant *H. pylori* (15). Metronidazole (Mtz) was a critical ingredient of the first successful therapy for *H. pylori* infection and remains a major component of newer triple and quadruple therapies (16, 21). Monotherapy with Mtz results in the acquisition of Mtz resistance by more than 50% of *H. pylori* isolates (31), and Mtz-containing therapies are being undermined by the development of resistance (36, 40).

Mtz has action against a wide variety of prokaryotic and eukaryotic pathogens including *H. pylori* and is a mainstay of therapy for infections with organisms such as *Bacteroides* species, *Clostridium* species, *Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba histolytica* (8, 33). Understanding of the antimicrobial action and resistance to Mtz has come from studies with anaerobic microorganisms such as *Bacteroides*, *Trichomonas*, and *Clostridium* species (8, 9, 30). The cytotoxicity of Mtz is not directly due to the final products of Mtz reduction but to the unstable and/or less reduced intermediates that damage DNA, resulting in strand breakage, helix destabilization, unwinding, and cell death (5, 6). Reductive activation of Mtz depends on the redox system of the target cell. Any redox system that possesses a reduction potential more negative than that of Mtz will donate its electrons preferentially to Mtz (27). The direct donors of electrons in anaerobic bacteria are thought to be ferredoxin-like Fe-S electron transport proteins such as ferredoxin (10, 29). In anaerobic organisms, the redox potential is −430 to −460 mV, the typical value for ferredoxin-like Fe-S proteins, whereas Mtz has a reduction potential of −415 mV, making Mtz an efficient electron acceptor. The lowest redox potentials obtainable by aerobic organisms (e.g., *Escherichia coli*) are those of NAD or NADH (−320 mV) and NADP or NADPH (−324 mV), such that these organisms are intrinsically Mtz resistant as they are unable to reduce Mtz. However, under aerobic conditions, one electron step can result in reoxidization by oxygen to the original compound, producing inactive Mtz (8, 33). As noted above, the most important step in the antimicrobial action of Mtz in bacteria is the reductive activation of the nitro group of Mtz (which makes Mtz toxic), which is controlled by the redox system of the target cell.

It has been proposed that the mode of action of Mtz in *H. pylori* is similar to that in anaerobic bacteria, although the optimal in vitro culture conditions for this pathogen are microaerophilic (2, 25). In addition, ferredoxin and ferredoxin-like proteins have been identified from two complete *H. pylori* genomic sequences (1, 39). Putative Mtz nitroreductases in-

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TABLE 1. PCR primers used to amplify portions of *H. pylori* genes

Primer pair	Encoded protein (gene)	Nucleotide sequences[a]	Size (bp) of PCR fragment
FX-A[b] FX-B[c]	Ferredoxin (*fdxA*; HP0277[d])	5′-CATGTCATTATTGGTGAATG-3′ 5′-GGCTCGTTGCATGGGGATTT-3′	441
FXLK-A FXLK-B	Ferredoxin-like protein (*fdxB*; HP1508)	5′-ATGCTTGAAACTTCTAGCCA-3′ 5′-CTGGGGCGATGAAATAAAAG-3′	475
FLD-A FLD-B	Flavodoxin (*fldA*; HP1161)	5′-ATTGGTATTTTTTTGGGAC-3′ 5′-AAAAGTCTGATTCTAGCGGGG-3′	583
FLVN-A FLVN-B	NAD(P)H flavin oxidoreductase (*frxA*: HP0642)	5′-ACAAGTGGTTGCTTTACAGC-3′ 5′-GCCGCTGCCATCATCATGTT-3′	450
OXI-A OXI-B	Oxygen-insensitive nitroductase (*rdxA*; HP0954)	5′-GACAATTATTAAACGAGCGC-3′ 5′-CCTCCAATAATGCAACTATC-3′	460
OOR-A OOR-B	2-Oxoglutarate oxidoreductase (*oorD*; HP0588)	5′-ATGGCTAAAATGAGCGCTCC-3′ 5′-CGCATTTGGGTAAAGCCACG-3′	480
POR-A POR-B	Pyruvate oxidoreductase (*porD*; HP1109)	5′-ATGAAAGATTGGAACGAATT-3′ 5′-GCCATTGAGTGAGAGCGGTA-3′	364
URE-A URE-B	UreaseB (*ureB*; HP0072)	5′-CTTCTGCAATCAATCATGCG-3′ 5′-ATAGAAGCGTTCGCGTCACC-3′	717

[a] Nucleotide sequences were obtained from the complete *H. pylori* genome sequence (39).
[b] Forward primer.
[c] Backward primer.
[d] Designation in the complete *H. pylori* genome sequence.

clude ferredoxin (HP0277; FdxA), flavodoxin (HP1161; FldA), three ferredoxin-like proteins (HP1508 [which we named FdxB], HP0588 [δ subunit of 2-oxoglutarate oxidoreductase; OorDABC], and HP1109 [δ subunit of pyruvate ferredoxin oxidoreductase; PorCDAB]), NAD(P)H flavin oxidoreductase (HP0642; FrxA), and oxygen-insensitive NAD(P)H nitroreductase (HP0954; RdxA). OorDABC, PorCDAB, and FldA have been purified from *H. pylori*, and the possible involvement of those proteins in Mtz resistance has been described (20, 22, 23, 26). Furthermore, Mtz resistance in *H. pylori* has also been suggested to be related to efficient DNA repair exerted by the *recA* protein (3) and the decreasing oxygen-scavenging capability at the site of Mtz reduction in resistant *H. pylori* strains (38). The most convincing data regarding Mtz resistance in *H. pylori* relate to inactivation of the *rdxA* gene, which inactivates Mtz nitroreductase activity (14). However, other pathways that lead to Mtz resistance exist because Mtz resistance has been described in *H. pylori* strains with an intact *rdxA* gene (24). In addition, the inactivation of *rdxA* alone is insufficient to explain the heterogeneity of Mtz resistance among clinical *H. pylori* isolates (7, 42).

In this study, we present the rate of incidence and the heterogeneity of Mtz resistance among 544 clinical *H. pylori* isolates from the United States with the full range of Mtz resistance (Mtz MICs, 8 to ≥256 μg/ml). Putative *H. pylori* Mtz nitroreductases (e.g., FdxA, FdxB, FldA, FrxA, RdxA, OorD, and PorD) were inactivated to explore which gene or gene combinations are involved in Mtz resistance. We found that only the *fdxB*, *frxA*, and *rdxA* genes could be inactivated without causing a lethal effect on *H. pylori*. Mtz resistance was conferred by inactivation of *fdxB*, *frxA*, or *rdxA*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* ATCC 700392 (which is the same as *H. pylori* 26695 [39]) was obtained from American Type Culture Collection (Rockville, Md.), and all other *H. pylori* isolates ($n = 544$) were obtained from the Veterans Affairs Medical Center, Houston, Tex. The isolation of *H. pylori* strains from gastric biopsy specimens was performed as described previously (17). The isolated *H. pylori* strains including ATCC 700392 were routinely cultured on brain heart infusion (BHI; Difco, Detroit, Mich.) agar plates containing 7% horse blood in a microaerobic atmosphere (10% CO_2 and 5% O_2) at 37°C for 2 to 3 days. Rifampin-resistant *H. pylori* 1857 for conjugation was generated by selection of spontaneously resistant colonies on 7% horse serum–BHI agar plates supplemented with 100 μg of rifampin per ml. When needed, selection for chloramphenicol- or kanamycin-resistant *H. pylori* was performed by adding 10 μg of chloramphenicol per ml or 15 μg of kanamycin per ml to the 7% horse blood–BHI agar plate. *E. coli* cells (XL-Blue [Stratagene] or DH5α [Bethesda Research Laboratories, Inc.]) were cultured in Luria-Bertani (37) broth or agar plates for the amplification of plasmids.

Determination of Mtz MICs. The MICs for 544 *H. pylori* isolates were determined by twofold agar dilution. Agar dilution plates were prepared with Mueller-Hinton (MH) agar as the base medium. Aged sheep blood (2 weeks old) was added to the MH base medium at a concentration of 5%. An Mtz solution (Sigma Chemical Co., St. Louis, Mo.) was prepared in sterile distilled water and was added to the 5% sheep blood–MH base medium to achieve serial twofold concentrations of between 0.015 and 256 μg of Mtz per ml. Fresh *H. pylori* isolates (2 to 3 days culture) were prepared in saline to an optical density at 625 nm of between 0.38 and 0.4. With a Steers-type replicating device (Cathra [no longer in business]), 1 to 5 μl of the adjusted inoculum was delivered to the agar plates. All plates were incubated under CampyPak Plus conditions (Becton Dickinson BBL, Cockeysville, Md.) for 3 days. The MIC was defined as the lowest concentration of Mtz that completely inhibited the growth of the inoculum. Mtz-resistant *H. pylori* ATCC 43504 was used as a quality control organism. Any test in which the Mtz MIC for the quality control organism was outside the approved range (64 to 256 μg/ml) was completely discarded. The MICs for all *H. pylori* strains with inactivated *fdxB*, *frxA*, and *rdxA* genes were determined with 7% horse blood–BHI agar or 5% sheep blood–MH agar plates supplemented with 0.5, 1, 2, 4, 8, 16, 32, 64, 128, or 256 μg of Mtz per ml and incubated for 3 to 4 days. The measurement was repeated twice to confirm the results by using the same 7% horse blood–BHI medium supplemented with Mtz. The MIC for *E. coli* DH5α harboring *frxA* and/or *rdxA* genes was determined by growing cells on LB agar plates supplemented with 10, 20, 40, 80, 160, or 320 μg of Mtz per ml.

PCR amplification of portions of *fdxA*, *fdxB*, *fldA*, *frxA*, *rdxA*, *oorD*, *porD*, and *ureB* (as a control) from *H. pylori* and their in vitro inactivation mutagenesis. Portions of the genes that encode FdxA, FdxB, FldA, FrxA, RdxA, OorD, PorD, and UreB were amplified by PCR with PCR primer pairs, as shown in Table 1. The identity of each PCR-amplified fragment was confirmed by DNA sequencing, and the confirmed DNA fragments were inserted into the *Eco*RV restriction enzyme site of pBluescript SK(+) (Stratagene, La Jolla, Calif.). The insert DNA was digested with an appropriate restriction enzyme to inactivate the genes in vitro. A chloramphenicol resistance gene cassette (*cat*) (41) was inserted into the

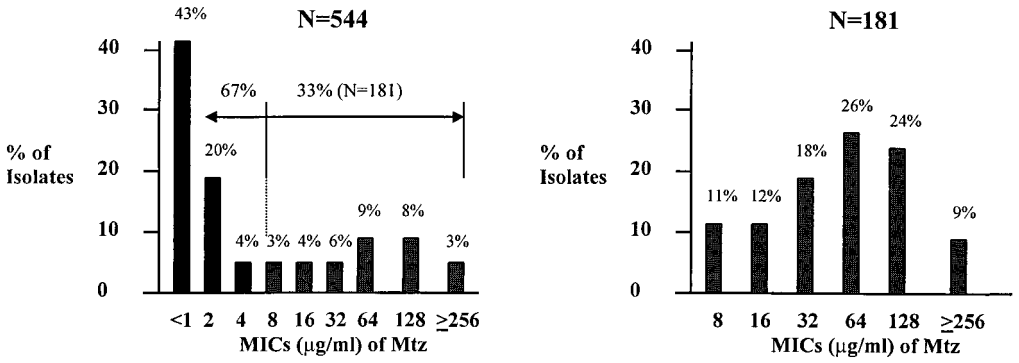

FIG. 1. Heterogeneity of Mtz resistance among clinical *H. pylori* isolates. (A) Distribution of Mtz MICs for clinical *H. pylori* isolates (n = 544). The MICs were determined by twofold agar dilution methods. A total of 181 of 544 isolates were Mtz resistant. (B) Distribution of Mtz MICs for Mtz-resistant *H. pylori* isolates (n = 181). The MICs were used to analyze the Mtz-resistant *H. pylori* isolates (181 of 544 isolates).

*Mun*I and *Bal*I sites of the insert DNAs for PorD and OorD, respectively. The *cat* cassette was also inserted into the *Bam*HI, *Nsi*I, and *Hin*dIII sites of the insert DNAs for FdxA, FrxA, and FldA, respectively, and into the *Eco*47III sites of the insert DNAs for FdxB and RdxA. The resulting plasmids were named pGH67 for the plasmid with *fdxA*::*cat*, pGH58 for that with *fdxB*::*cat*, pGH64 for that with *fldA*::*cat*, pGH130 for that with *frxA*::*cat*, pGH55 for that with *rdxA*::*cat*, pGH46 for that with *porD*::*cat*, and pGH60 for that with *oorD*::*cat*. A kanamycin resistance gene cassette from pHel3 (*km*) (19) was also inserted into the *Eco*47III site of insert DNA for RdxA, and the resulting plasmid (pGH87) was used for dual inactivation by selection on a plate with chloramphenicol and kanamycin. All the resulting plasmids (1 to 2 μg) were used for inactivation of chromosomal genes by natural transformation as described previously (19).

Cloning of *frxA* and *rdxA* genes from *H. pylori* and DNA sequence analysis. To isolate the *frxA* and *rdxA* genes from *H. pylori* ATCC 700392, 2600, 6013, 1857, and 1700, lambda phage genomic libraries were constructed with genomic DNAs from the strains as described previously (12). The *frxA*- and *rdxA*-positive phage clones from each genomic library were screened by plaque hybridization with the *frxA*- and *rdxA*-specific PCR clones described above. The appropriate restriction fragments from the screened phage clones carrying the *frxA* and *rdxA* genes were identified by hybridization with the same probes and inserted into pBluescript SK(+) or *H. pylori-E. coli* shuttle vector pHel2 (19). The cloned *frxA* and *rdxA* genes from each *H. pylori* strain were used for DNA sequencing or complementation of Mtz sensitivity. The DNA sequences of both DNA strands of the cloned genes were determined at the Molecular Genetics Facility at the Baylor College of Medicine. DNA sequence analysis was accomplished by the BLAST network service of the National Center for Biotechnology Information.

Complementation of Mtz sensitivity. For complementation of an Mtz-sensitive strain to an Mtz-resistant strain, plasmid DNA [1 to 2 μg; the cloning vector was pBluescript SK(+), which is not replicated in *H. pylori*] that carried naturally inactivated *frxA* or *rdxA* genes was introduced into Mtz-sensitive *H. pylori* 2600 by natural transformation (19). Transformed *H. pylori* 2600 was screened on a 7% horse blood–BHI agar plate supplemented with 4 μg of Mtz per ml. For the complementation of an Mtz-resistant strain to an Mtz-sensitive strain, the functional *frxA* and/or *rdxA* gene from an Mtz-sensitive *H. pylori* 2600 isolate was cloned into *H. pylori-E. coli* shuttle vector pHel2. The cloned *frxA* and/or *rdxA* gene in pHel2 was introduced into rifampin-resistant *H. pylori* strain 1857 (Mtz MIC, 128 μg/ml) by triparental conjugation (19), and the conjugated *H. pylori* colonies (10 of each) were used to measure Mtz sensitivity.

Mtz nitroreductase enzyme assay. *E. coli* XL-1 Blue carrying the cloned *frxA* and *rdxA* genes was aerobically cultured to the late log phase in LB broth to measure Mtz nitroreductase activity. The cells were harvested in phosphate-buffered saline containing 1 mM dithiothreitol (4°C) to protect oxygen-sensitive enzymes and subjected to French pressure (600 lb; Aminco, Urbana, Ill.). The cell-free crude extracts were centrifuged at 15,000 × *g* for 15 min at 4°C to remove the cell debris, and the supernatant was immediately used as the enzyme source. The Mtz nitroreductase activity from the cells was measured by the method of Goodwin et al. (14). All enzyme reactions were performed at 25°C in 1-ml volumes in triplicate, and enzymatic activities were calculated as nanomoles per minute per milligram of protein. The protein concentrations of the crude cell extracts were determined by the Bradford procedure (Bio-Rad) with bovine serum albumin as the standard.

Nucleotide sequence accession numbers. The GenBank accession numbers for the *frxA* genes are AF183174 for strain 2600, AF1833992 for strain 6013, AF183176 for strain 1857, and AF183175 for strain 1700. The GenBank accession numbers for the *rdxA* genes are AF184266 for strain 2600, AF184268 for strain 6013, AF184269 for strain 1857, and AF184267 for strain 1700.

RESULTS

Analysis of Mtz resistance among clinical *H. pylori* isolates. The heterogeneity of Mtz resistance in a single or multiple colony expansion was demonstrated with 12 *H. pylori* strains isolated from duodenal ulcer patients (7). To understand the variations in the MICs for clinical *H. pylori* isolates, we determined the MICs for 544 *H. pylori* strains from the Veterans Affairs Medical Center, Houston, Tex., using the agar dilution method as described above. Since strain ATCC 700392 was considered Mtz sensitive (14), we carefully repeated the MIC measurement using both the agar dilution method and the E-test as described previously (18). The Mtz MIC for *H. pylori* ATCC 700392 was repeatedly 8 μg/ml by the agar dilution method and 16 μg/ml by the E-test. The MICs for 544 clinical *H. pylori* isolates revealed that 33% were Mtz resistant (181 of 544 isolates; Mtz MICs, ≥8 μg/ml) and 67% were Mtz sensitive strains (363 of 544 isolates; Mtz MICs, ≤4 μg/ml), with a wide spectrum in the MICs (8 to ≥256 μg/ml) among Mtz-resistant strains (Fig. 1).

Inactivation analysis of genes encoding putative Mtz nitroreductases (*fdxA*, *fdxB*, *fldA*, *frxA*, *rdxA*, *oorD*, *porD*) using genetic transformation of clinical *H. pylori*. We identified genes from the complete *H. pylori* genomic DNA sequence (e.g., *fdxA*, *fdxB*, *fldA*, *frxA*, *rdxA*, *oorD*, and *porD*) that encode putative Mtz nitroreductases. We evaluated the natural competence and transformation frequencies of 50 strains (25 Mtz-sensitive and 25 Mtz-resistant strains including *H. pylori* ATCC 700392) selected from among the 544 clinical *H. pylori* isolates. As a control gene for natural transformation, we chose the *ureB* gene, which is not essential for *H. pylori* survival (11). The amino terminus (717 bp) of the *ureB* gene from *H. pylori* ATCC 700392 was amplified with a PCR primer pair (URE-A–URE-B; Table 1), and the PCR-amplified *ureB* gene was inactivated by inserting a chloramphenicol resistance cassette (*cat*) (41). The plasmid that contained the inactivated *ureB* gene (pUre1) was used for inactivation of the *H. pylori* chromosomal *ureB* gene. Of the 50 clinical *H. pylori* isolates, 18 strains (7 Mtz-sensitive and 11 Mtz-resistant strains) were able to take up plasmid pUre1, as determined by expression of the chloramphenicol resistance marker in the progeny *H. pylori*, when it was applied by natural transformation (19). The inactivation of *ureB* in the chromosomal DNA was confirmed by PCR amplification of the *ureB* gene from parental and mutant *H. pylori* strains following Southern blot hybridization as described pre-

TABLE 2. PCR primers used to confirm whether the *H. pylori* genes were interrupted

Primer pair	Encoded protein (gene)	Nucleotide sequence[a]	Size (bp) of PCR fragment
FXLK-A1[b] FXLK-B1[c]	Ferredoxin-like protein (*fdxB*; HP1508[d])	5′-CCTAAAATGCTAGCGATAGC-3′ 5′-ATCAAACAAGGCTTGCCTTA-3′	1,850
FLVN-A1 FLVN-B1	NAD(P)H flavin oxidoreductase (*frxA*: HP0642)	5′-CAAAGCTTGGGTTACCAGCACC-3′ 5′-CCGCTTCCGCGTTTTGCTTCGTA-3′	1,254
OXI-A1 OXI-B1	Oxygen-insensitive nitroreductase (*rdxA*; HP0954)	5′-AAGCTTTTGATTTATTTGGA-3′ 5′-CTTTAATTTAGGTTTGATTA-3′	1,130
URE-A1 URE-B1	Urease B (*ureB*; HP0072)	5′-ATGAAAAAGATTAGCAGAAA-3′ 5′-CTAGAAAATGCTAAAGAGTT-3′	1,710

[a] Nucleotide sequences were obtained from the complete *H. pylori* genome sequence.
[b] Forward primer.
[c] Backward primer.
[d] Designation in the complete *H. pylori* genome sequence.

viously (28) and by the urease-negative activities of the *ureB* mutant *H. pylori* strains. The transformation frequencies of the 18 *H. pylori* isolates ranged from 4×10^{-3} (strain 2600) to 9×10^{-6} (strain 2399) viable cells with plasmid pUre1. Thirty-two of the 50 *H. pylori* isolates were nontransformable with pUre1. The 32 nontransformable strains were also tested for natural competence by the method of Wang et al. (41), and the antibiotic resistance markers from pUre1 failed to be introduced into these strains. We used the 18 transformable *H. pylori* strains for the inactivation of the genes that encode putative Mtz nitroreductases. To test whether the genes were inactivated without a lethal effect, in vitro inactivated genes (by the *cat* gene) that encode putative Mtz nitroreductases (pGH67 for *fdxA*, pGH58 for *fdxB*, pGH64 for *fldA*, pGH130 for *frxA*, pGH55 for *rdxA*, pGH60 for *oorD*, pGH46 for *porD*) were introduced into *H. pylori* 2600. The results revealed that only *fdxB*, *frxA*, and *rdxA* were inactivated without a lethal effect on *H. pylori* 2600. Inactivation of all the other genes (*fdxA*, *fldA*, *oorD*, and *porD*) to produce viable cells failed when the inactivation was repeated by the transformation method of either Heuermann and Haas (19) or Wang et al. (41), suggesting that the cells were nonviable as a result of the inactivation. The *rdxA* inactivation and the lethal effect of *oorDABC* and *porCDAB* inactivation have been reported previously (14, 22).

Mtz sensitivity analysis of *H. pylori* strains in which *fdxB*, *frxA*, and *rdxA* are inactivated. Although the involvement of the null mutation in the *rdxA* gene in Mtz resistance has been shown (14), inactivation of *rdxA* results in a narrow range of MICs (e.g., 16 to 32 μg/ml), which is different from the wide range of MICs shown for clinical *H. pylori* isolates. To assess the roles of the *fdxB*, *frxA*, and *rdxA* genes in Mtz resistance, we inactivated the *fdxB*, *frxA*, and *rdxA* genes using the 18 transformable *H. pylori* strains (7 Mtz-sensitive and 11 Mtz-resistant strains). In addition, we also inactivated the *fdxB* or *frxA* with the *rdxA* genes (dual inactivation). Inactivation of one or both genes was confirmed by PCR amplification following Southern blot hybridization as described previously (28). To avoid chloramphenicol- or kanamycin-resistant *H. pylori* mutants that contained a single crossover during homologous recombination (i.e., false-positive recombination), new PCR primer pairs positioned 193 to 960 bp away from the positions of the sequences of the PCR primer pairs for the PCR clones used for inactivation were designed (Table 2). The integrities of the mutant genes were then reconfirmed by PCR amplification with the new PCR primer pairs. The integrity of the mutant phenotype (antibiotic resistance) was also confirmed with 10 colonies isolated from each mutant strain. All the confirmed mutant strains were then analyzed for Mtz sensitivity (Table 3 and Table 4). The MICs for all mutant strains were determined simultaneously, and the results were confirmed twice. The MIC for a strain that had an inactivated *fdxB* gene and that was constructed from the Mtz-sensitive strains was not different from those for the parental strains. The MICs for strains that had inactivated *rdxA* genes and that were constructed from seven Mtz-sensitive strains were increased to 32 μg/ml for six strains, to 128 μg/ml for one strain (strain 2600). The MICs for the same seven strains but with inactivated *frxA* genes were also increased to the same levels as those for the strains with

TABLE 3. Mtz sensitivity analysis of Mtz-sensitive *H. pylori* strains with inactivated *fdxB*, *frxA*, and *rdxA* genes

H. pylori strain	MIC (μg/ml) for the clinical isolate or mutant strain[a]:							
	Clinical isolates	*fdxB*::*cat*[b]	*frxA*::*cat*	*rdxA*::*cat*	*ureB*[c]::*cat*	*fdxB*::*cat rdxA*::*km*[d]	*frxA*::*cat rdxA*::*km*	*ureB*::*cat rdxA*::*km*
2714	1	1	32	32	1	64	128	32
2393	1	4	32	32	1	64	128	32
2667	1	2	32	32	1	64	128	32
2600	2	2	128	128	2	128	>256	128
2201	2	2	32	32	2	64	128	32
2399	4	2	32	32	2	64	128	32
2418	4	2	32	32	2	64	128	32

[a] MICs were determined by growth on 7% horse blood–BHI agar plates supplemented with 0.5, 1, 2, 4, 8, 16, 32, 64, 128, or 256 μg of Mtz per ml and incubation for 4 days. The results were confirmed twice by measuring all MICs at the same time.
[b] *cat*, chloramphenicol resistance gene.
[c] *ureB*::*cat* was used as a negative control.
[d] *km*, kanamycin resistant gene.

TABLE 4. Mtz sensitivity analysis of Mtz-resistant *H. pylori* strains with inactivated *fdxB*, *frxA*, and *rdxA* genes

H. pylori strain	MIC (μg/ml) for the clinical isolate or mutant strain[a]:			
	Clinical isolates	*fdxB*::*cat*	*frxA*::*cat*	*rdxA*::*cat*
ATCC 700392	8	64	32	64
2617	8	32	64	64
2593	8	32	64	64
2620	32	64	128	64
6013	32	64	32	128
2723	64	128	128	128
9004	64	128	128	128
9002	128	ND[b]	128	128
1857	128	ND	128	128
1700	256	ND	256	256
7200	256	ND	256	256

[a] MICs were determined by growth on 7% horse blood–BHI agar plates supplemented with 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 μg of Mtz per ml and incubation for 4 days. The results were confirmed twice by measuring all MICs at the same time.
[b] ND, not determined.

inactivated *rdxA* genes, irrespective of the slower growth rate of the strains inactivated with the *frxA* genes. Interestingly, the MICs for the seven Mtz-sensitive strains with the *rdxA-fdxB* dual inactivation increased to 64 μg/ml (i.e., greater than that for strains with either an inactivated *rdxA* gene or an inactivated *fdxB* gene) for six strains and to 128 μg/ml for one strain (strain 2600). These results are consistent with the notion that *fdxB* inactivation is involved in Mtz resistance but at a lower level than the level of involvement of *rdxA* inactivation. In addition, the MICs for the seven Mtz-sensitive strains with the *frxA-rdxA* dual inactivation increased to 128 μg/ml Mtz for six strains; the MIC for one strain (strain 2600) increased to >256 μg/ml (Table 3).

We also analyzed the Mtz sensitivities of mutant strains constructed from Mtz-resistant strains for which Mtz MICs were between 8 and 64 μg/ml. The MICs for the strains that had inactivated *fdxB* genes and that were constructed from these resistant strains increased up to eightfold, which provided additional evidence for the involvement of *fdxB* inactivation in the Mtz resistance of *H. pylori*. The MICs for the strains that had inactivated *frxA* or *rdxA* genes and that were constructed from strains with low or moderate levels of resistance also increased up to eightfold, suggesting that multiple genes or factors are involved in the wide spectrum of Mtz resistance. Interestingly, the MIC for strain 6013 (32 μg/ml) was not changed when the *frxA* gene was inactivated, whereas it increased fourfold when the *rdxA* gene was inactivated, suggesting that strain 6013 contains a nonfunctional *frxA* gene and a functional *rdxA* gene. Additionally, the MICs for strains 9002 and 1857 (128 μg/ml) and strains 1700 and 7200 (256 μg/ml) did not change because of either *frxA* or *rdxA* inactivation, suggesting that the strains may contain both nonfunctional *frxA* and nonfunctional *rdxA* genes in their genomes (Table 4).

Cloning and nucleotide sequence analysis of *frxA* and *rdxA* genes from *H. pylori* strains. To prove that naturally inactivated *frxA* genes are present in clinical Mtz-resistant *H. pylori* isolates, the *frxA* and *rdxA* genes were cloned from Mtz-resistant strains and the nucleotide sequences were analyzed. Because the DNA sequences of the PCR clones may not always be identical to the parental DNA sequence, we constructed lambda phage libraries using genomic DNAs purified from Mtz-resistant strains ATCC 700392, 6013, 1857, and 1700 and Mtz-sensitive strain 2600. *frxA*- and *rdxA*-positive clones were isolated from each genomic library, and the restriction enzyme maps of the initial clones are shown in Fig. 2. The restriction enzyme sites were highly diverse among the clones except in the *frxA*- and the *rdxA*-coding regions, reflecting the genomic DNA sequence diversity in *H. pylori* strains. The DNA sequences of the *frxA* and *rdxA* genes were determined by using

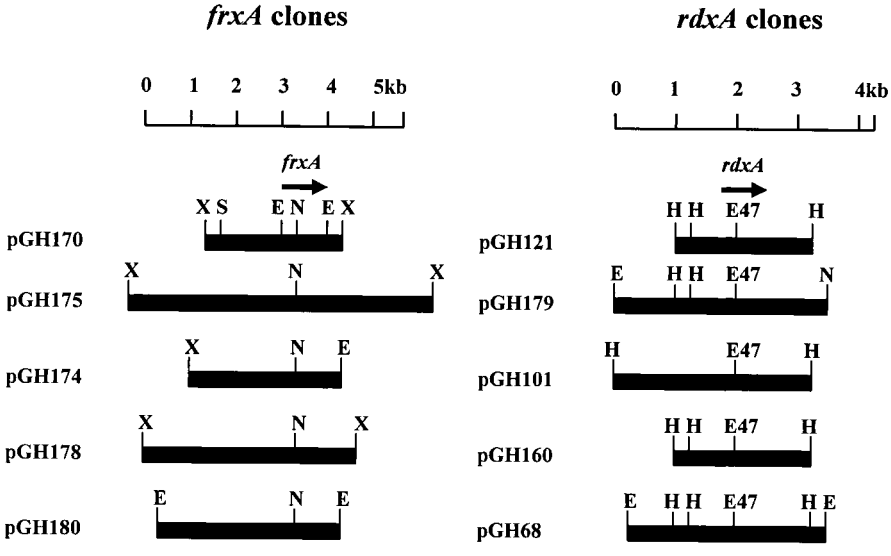

FIG. 2. Restriction endonuclease map of *frxA* and *rdxA* clones from the lambda phage library constructed with genomic DNAs from Mtz-sensitive and -resistant *H. pylori* strains. The phage clones that carried an *frxA* gene or an *rdxA* gene were screened by plaque hybridization. Restriction fragments that contained an *frxA* or an *rdxA* gene were identified by Southern hybridization. The restriction fragments that contained the *frxA* or *rdxA* gene were inserted into pBluescript SK(+) digested with the same or appropriate restriction enzymes. pGH170 and pGH121 were cloned from the genomic DNA of *H. pylori* 2600 (Mtz MIC, 2 μg/ml), pGH175 and pGH179 were cloned from the genomic DNA of *H. pylori* ATCC 700392 (Mtz MIC, 8 μg/ml), pGH174 and pGH101 were cloned from the genomic DNA of *H. pylori* 6013 (Mtz MIC, 32 μg/ml), pGH178 and pGH160 were cloned from the genomic DNA of *H. pylori* 1857 (Mtz MIC, 128 μg/ml), and pGH180 and pGH68 were cloned from the genomic DNA of *H. pylori* 1700 (Mtz MIC, 256 μg/ml). E, *Eco*RI; E47, *Eco*47III; H, *Hin*dIII; N, *Nsi*I; S, *Sph*I; X, *Xba*I.

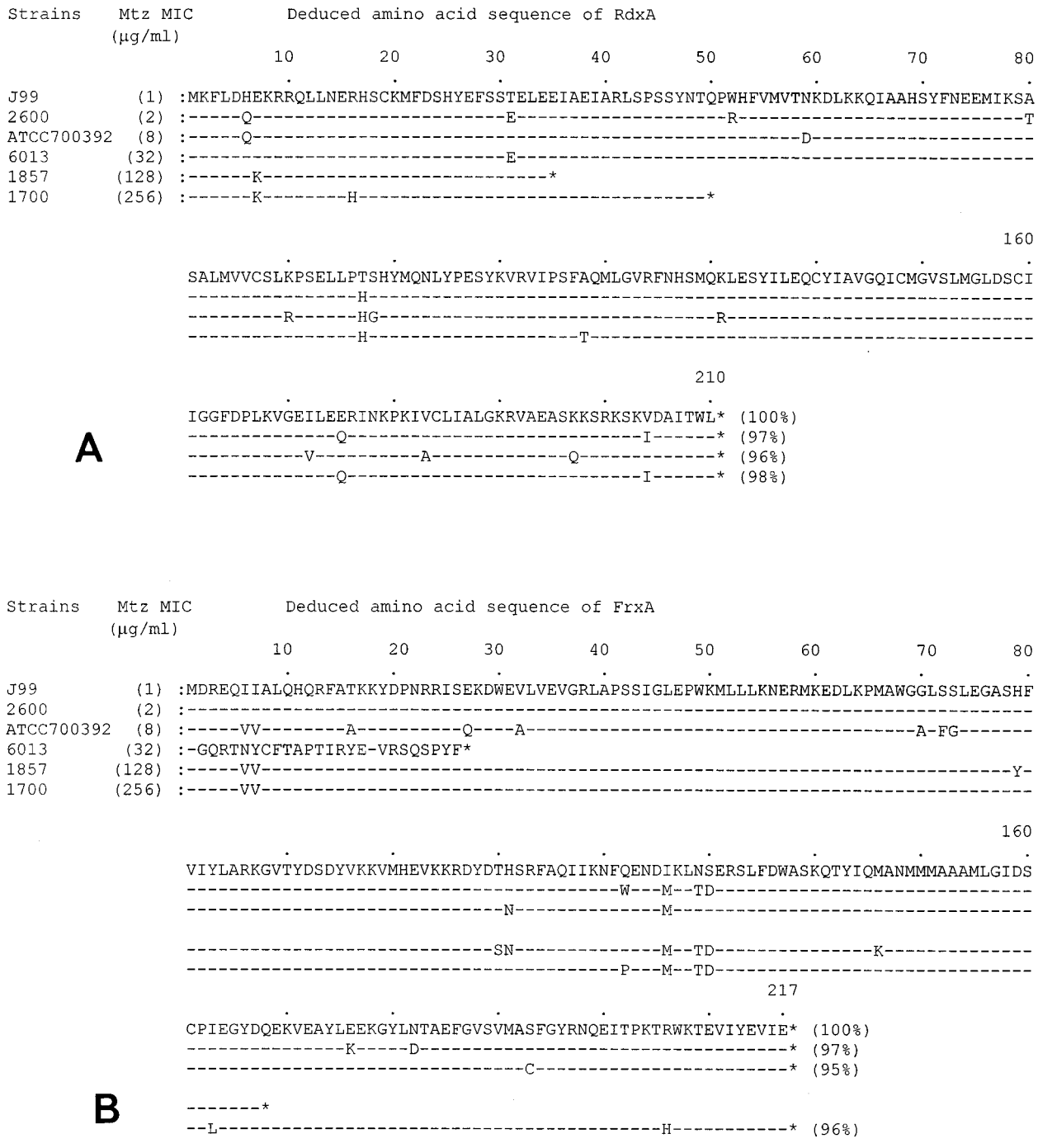

FIG. 3. Alignment of RdxA (A) and FrxA (B) amino acid sequences from Mtz-sensitive and -resistant *H. pylori* strains. Percentages in parentheses are percent identity.

subclones of the initial clones, and the deduced amino acid sequences of the genes were in alignment, as shown in Fig. 3. Nucleotide sequence analysis of an ~1.0-kb *Eco*RI fragment from pGH170 (cloned from strain 2600) revealed that the 971-bp fragment contained an *frxA* gene with 97% identity compared to the sequence of *frxA* from strain J99 (Fig. 3B). Upstream of the *frxA* gene was the carboxyl terminus (78 of 285 amino acids) of the putative 3-hydroxyacid dehydrogenase gene, with only 2 bp of intervening nucleotides. The FrxA proteins from resistant strains 6013 and 1857 were truncated by insertion of one nucleotide (G) in the FrxA-coding region (between the 1st and 2nd amino acids), which shifted a reading frame of FrxA, and a nonsense mutation (168th amino acid, CAA [Gln] to TAA [stop codon]), respectively (Fig. 3B). However, the FrxA protein from resistant strain 1700 was not truncated and showed 97% identity compared with the sequence of the FrxA protein from Mtz-sensitive strain J99 (Mtz MIC, 1 μg/ml) (1; Richard A. Alm, personal communication). The

TABLE 5. Complementation analysis of Mtz sensitivity with cloned *frxA* and *rdxA* genes

H. pylori strain and clone	Transformation frequency[a] (CFU/μg of plasmid DNA)
No DNA	None[b]
H. pylori 2600 (Mtz MIC, 2 μg/ml)	
pGH170 (*frxA*)	None
pGH121 (*rdxA*)	None
H. pylori 6013 (Mtz MIC, 32 μg/ml)	
pGH174 (*frxA*)[c]	0.5×10^3
pGH101 (*rdxA*)	None
H. pylori 1857 (Mtz MIC, 128 μg/ml)	
pGH160 (*frxA*)[d]	0.58×10^3
pGH178 (*rdxA*)[d]	0.52×10^3
H. pylori 1700 (Mtz MIC, 256 μg/ml)	
pGH180 (*frxA*)	None
pGH68 (*rdxA*)[d]	0.5×10^4

[a] Approximately 3×10^7 cells (optical density at 550 nm = 0.1) of *H. pylori* 2600 were used for natural transformation by applying plasmid DNA (1 μg) purified from *E. coli* DH5α, and Mtz-resistant *H. pylori* 2600 was screened on BHI-horse blood agar plates supplemented with 4 μg of Mtz per ml (the values are the averages of two experiments).

[b] Spontaneous Mtz-resistant cells (two or three colonies) were detected, and these cell numbers were subtracted from the number of transformants.

[c] FrxA was truncated by insertion of one nucleotide (G) in the coding region (see text).

[d] FrxA or RdxA was truncated by nonsense mutations (see text).

FrxA protein from low-level Mtz-resistant strain ATCC 700392 showed 95% identity compared with the sequence of the FrxA protein from strain J99. On the other hand, the RdxA proteins from resistant strains 1857 and 1700 were truncated by nonsense mutations. However, the RdxA protein from resistant strain 6013 was not truncated and showed 98% identity compared with the sequence of the RdxA protein from Mtz-sensitive strain J99. The RdxA protein from low-level Mtz resistant strain ATCC 700392 showed 96% identity compared with the sequence of the RdxA protein from strain J99 (Fig. 3A).

Complementation analysis of Mtz sensitivity using cloned *frxA* and *rdxA* genes in *H. pylori*. It has been shown that the Mtz resistance of *H. pylori* can be transferred from a resistant strain to a sensitive strain by introduction of genomic DNA from a resistant strain into a sensitive strain (20, 41). On the basis of the results presented above, the Mtz resistance acquired by the sensitive strain may be due to replacement of the inactivated *rdxA* and/or *frxA* genes from the resistant strain. We examined whether the naturally inactivated *frxA* genes from Mtz-resistant strains were able to transfer Mtz resistance to Mtz-sensitive strains. As shown in Table 5, plasmid DNA that contained naturally inactivated *frxA* genes from Mtz-resistant strains 6013 and 1857 (pGH174 and pGH160, respectively) successfully transferred Mtz resistance to Mtz-sensitive strain 2600 with transformation frequencies of 0.5×10^3 and 0.58×10^3 CFU per 1 μg of plasmid DNA, respectively. In the same complementation study, the naturally inactivated *rdxA* genes from Mtz-resistant strains 1857 and 1700 (pGH178 and pGH68, respectively) also transferred Mtz resistance to Mtz-sensitive strain 2600 with transformation frequencies of 0.52×10^3 and 0.5×10^4 CFU per 1 μg of plasmid DNA, respectively. However, none of the plasmid DNAs that contained functional *frxA* or *rdxA* genes (pGH170, pGH121, pGH101, and pGH180) transferred Mtz resistance to Mtz-sensitive strain 2600. We also introduced a functional *frxA* and/or *rdxA* gene cloned in the *H. pylori-E. coli* shuttle vector (pHel2) into one of the Mtz-resistant strains to confirm whether the functional genes were able to restore the Mtz sensitivities of the strains. Mtz resistance in strain 1857 (Mtz MIC, 128 μg/ml) was partially decreased by a functional *frxA* gene (pGH177) or a functional *rdxA* gene (pGH127), but it was made completely susceptible (MIC, 1 μg/ml) when both functional *frxA* and *rdxA* genes (pGH181) were introduced. These results are consistent with the notion that the *frxA* inactivation is involved in Mtz resistance.

Expression of cloned *frxA* and *rdxA* genes in *E. coli*. We performed the Mtz nitroreductase assay using *E. coli* strains that harbored cloned *frxA* genes or an *rdxA* gene (as a positive control) to verify whether the cloned *frxA* gene from the Mtz-sensitive *H. pylori* strain possessed Mtz nitroreductase activity. Mtz nitroreductase activity was always demonstrable in the *E. coli* strains that harbored a cloned *rdxA* gene from the Mtz-sensitive strain *H. pylori* 2600, with the enzyme activity varying between 5.3 and 7.8 nmol/mg/min. By the same assay Mtz nitroreductase activity was not detected in the *E. coli* strains that harbored a cloned *frxA* gene from Mtz-sensitive strain *H. pylori* 2600. The difficulty in measuring Mtz nitroreductase activity in crude extracts may be due to oxidation of key components during the preparation of crude extracts or the interference of endogenous nitroreductase from *E. coli* as described by Goodwin et al. (14).

An alternative method for detection of Mtz nitroreductase activity in *E. coli* is an in vivo assay, which measures the MICs for *E. coli* strains that harbor a cloned *frxA* or *rdxA* gene from *H. pylori*. The in vivo assay is based on expression of a cloned *frxA* or *rdxA* gene in *E. coli* cells and measurement of the Mtz sensitivities of the *E. coli* cells that harbor the cloned genes. If the cloned gene expresses and produces Mtz nitroreductase in intrinsically Mtz-resistant *E. coli*, the enzyme should convert nontoxic Mtz to toxic Mtz and the *E. coli* cells will become sensitive to Mtz (decreased MICs). Since *E. coli* DH5α is intrinsically Mtz resistant (MIC, >320 μg/ml), we introduced the cloned *frxA* and *rdxA* genes from Mtz-sensitive and -resistant *H. pylori* strains. The *E. coli* DH5α cells that harbored the genes were cultured aerobically in LB broth and spotted (5 μl of each strain) onto LB agar plates supplemented with 10 to 320 μg of Mtz per ml. The plates were then incubated aerobically at 37°C for 18 h. The *E. coli* DH5α cells that harbored either the *frxA* or the *rdxA* gene cloned from Mtz-sensitive strain *H. pylori* 2600 (pGH170 or pGH121, respectively) did not grow in the presence of 20 to 40 μg of Mtz per ml. In contrast, only the vector [pBluescript SK(+) or pHel2] or plasmid that contained a part of the *frxA* or the *rdxA* gene (pGH172, *Xba*I-*Nsi*I fragment of pGH170; pGH104, *Eco*47III-*Hin*dIII fragment of pGH121; see Fig. 2) grew on medium with 320 μg of Mtz per ml. Interestingly, *E. coli* cells that harbored the *Sph*I-*Xba*I fragment (2.5 kb; pGH173) of pGH170 also grew on medium with 320 μg of Mtz per ml (Table 6). *E. coli* cells that harbored the same *frxA* or *rdxA* gene in the *H. pylori-E. coli* shuttle vector (pHel2) were also sensitive to Mtz, but the MICs for the strains were slightly higher (MICs, 40 to 80 μg/ml for strains with pGH127 and pGH177) than those obtained when pBluescript SK(+) was used as a cloning vector. In addition, *E. coli* cells that harbored both genes (*frxA* and *rdxA*) in pHel2 (pGH181) did not grow on LB agar plates supplemented with 20 to 40 μg of Mtz per ml. Although the MICs were slightly variable in the in vivo assay, the results were reproducible and consistent. Therefore, we applied the assay to the other cloned *frxA* and *rdxA* genes from Mtz-resistant *H. pylori* strains. *E. coli* cells that harbored a *frxA* gene (pGH175) or a *rdxA* gene (pGH179) from *H. pylori* ATCC 700392 did not

TABLE 6. MICs for *E. coli* cells expressing cloned *frxA* and *rdxA* genes

H. pylori strain and clone	Mtz MIC (μg/ml)[a]
pBluescript SK(+)[b]	>320
pHel2[c]	>320
H. pylori 2600 (Mtz MIC, 2 μg/ml)	
pGH170 (*frxA*)	40
pGH121 (*rdxA*)	40
pGH177 (*frxA*)	80
pGH127 (*rdxA*)	80
pGH181 (*frxA*/*rdxA*)	40
pGH172 (Δ*frxA*)[d]	>320
pGH173 (*frxA*)[d]	>320
pGH104 (Δ*rdxA*)[e]	>320
H. pylori ATCC 700392 (Mtz MIC, 8 μg/ml)	
pGH175 (*frxA*)	160
pGH179 (*rdxA*)	40
H. pylori 6013 (Mtz MIC, 32 μg/ml)	
pGH174 (*frxA*)[f]	>320
pGH101 (*rdxA*)	40
H. pylori 1857 (Mtz MIC, 128 μg/ml)	
pGH160 (*frxA*)[g]	>320
pGH178 (*rdxA*)[g]	>320
H. pylori 1700 (Mtz MIC, 256 μg/ml)	
pGH180 (*frxA*)	40
pGH68 (*rdxA*)[g]	>320

[a] Overnight cultures (5 μl) of *E. coli* DH5α harboring each clone were spotted onto LB agar plates supplemented with 10, 20, 40, 80, 160, or 320 μg of Mtz per ml, and the plates were incubated for 18 h. The measurement was repeated three times with identical results.

[b] pBluescript SK(+) was used as a cloning vector for all clones except pGH127, pGH177, and pGH181.

[c] pHel2 was used as a cloning vector for clone pGH127, pGH177, and pGH181, which carried *frxA* or *rdxA* from pGH170 or pGH121, respectively, to introduce the clones into *H. pylori* 1857.

[d] pGH172 and pGH173 originated from pGH170.

[e] pGH104 originated from pGH121.

[f] FrxA was truncated by insertion of one nucleotide (G) in the coding region (see text).

[g] FrxA or RdxA were truncated by nonsense mutations (see text).

grow on LB agar plates supplemented with 80 to 160 and 20 to 40 μg of Mtz per ml, respectively. Repetition of these assays with pGH175 and pGH179 gave identical results. We also confirmed the involvement of an *frxA* gene from strain ATCC 700392 in Mtz sensitivity by comparing the expression of a part of the *frxA* gene (*Xba*I-*Nsi*I fragments of pGH175; Fig. 2) that, when expressed in *E. coli*, resulted in loss of Mtz nitroreductase activity of the gene. *E. coli* cells that harbored *frxA* (pGH174 and pGH160) or *rdxA* (pGH178 and pGH68) from Mtz-resistant strains 6013, 1857, and 1700 grew on LB agar plates supplemented with 320 μg of Mtz per ml, while *E. coli* cells that harbored *frxA* (pGH180) and *rdxA* (pGH101) from Mtz-resistant strains 6013 and 1700 did not grow on LB agar plates supplemented with 20 to 40 μg of Mtz per ml (Table 6).

DISCUSSION

H. pylori infection is responsible for most cases of peptic ulcer disease, and successful treatment of the infection results in cure of the disease. In the last decade, a number of regimens for the treatment of *H. pylori* infection have been introduced. Mtz resistance among *H. pylori* isolates has been found worldwide and has become an increasing problem for current therapies. The deciphering of the Mtz resistance mechanism may provide critical information for (i) the appropriate antibiotic treatment of this infection, (ii) better therapy for infections caused by Mtz-resistant *H. pylori* strains and perhaps for those caused by other Mtz-resistant microorganisms, and (iii) the design of new antibiotics. The mechanism of Mtz resistance in *H. pylori* was initially explained by mutations in an *rdxA* gene (14). However, as shown here and by Jenks et al. (24), an intact *rdxA* gene can be found in some Mtz-resistant strains, suggesting that an additional resistance mechanism(s) is involved in Mtz resistance. To investigate whether additional Mtz resistance mechanisms were present in *H. pylori*, we examined the nature of Mtz resistance among 544 clinical *H. pylori* isolates, clarified the role of an *rdxA* gene in a wide range of Mtz-resistant *H. pylori* isolates, and explored additional genes that might be involved in Mtz resistance. The 33% rate of Mtz resistance found in this study is in agreement with the rates detected by other investigators (13, 32). The proposed breakpoint for Mtz resistance used in this study was an MIC of ≥8 μg/ml, which is based on the finding that inactivation of the *rdxA* or *frxA* genes of *H. pylori* strains for which the MICs are ≤4 μg/ml always increased the MICs to 32 μg/ml but inactivation of strains for which the MICs are ≥8 μg/ml increased the MICs to >32 μg/ml. Inactivation of *fdxB* in strains for which the MICs are ≥8 μg/ml also increased the MICs compared with those for the parental strains. These results suggest that acquired Mtz resistance begins at an MIC of approximately ≥8 μg/ml. Mtz MICs for Mtz-sensitive strain 2600 fluctuated from <1 to 4 μg/ml, but the strain never grew in the presence of 4 μg of Mtz per ml (MIC, <8 μg/ml).

Inactivation of *rdxA* in Mtz-sensitive strains always increased the MIC to 32 μg/ml. For the Mtz-sensitive strains with inactivated *rdxA* genes, the MICs were never lower than 32 μg/ml for any of the isolates, suggesting that complete *rdxA* inactivation may generally increase the MIC to 32 μg/ml (moderate level of Mtz resistance). For one strain (strain 2600), inactivation of *rdxA* increased the Mtz MIC to 128 μg/ml (high-level resistance), suggesting that *rdxA* inactivation may play a role in high-level Mtz resistance, although the possibility that an additional factor(s) or a lack of a factor(s) may also be involved in high-level Mtz resistance. This observation was also shown in high-level Mtz-resistant clinical isolate 1700, in which only *rdxA* was inactivated.

Theoretically, any protein that produces or inhibits Mtz nitroreductase activity could be involved in Mtz sensitivity. Purified PorCDAB and FldA proteins were tested in vitro and the results suggested that these proteins are putative Mtz nitroreductases (23, 26). Inactivation of PorCDAB was lethal to *H. pylori* (22), and we also confirmed that inactivation of the *porCDAB* or *fldA* gene was lethal to *H. pylori*. The inactivation of other ferredoxin-like or -linked proteins (FdxA and OorD) that may have Mtz nitroreductase activities was also lethal to *H. pylori*. Since FdxA, FldA, PorCDAB, and OorDABC appeared to be essential for cell survival, it was difficult to assess the roles of these proteins in Mtz resistance. Although inactivation of a single gene (*fdxB*) in an Mtz-sensitive strain had no effect on the MIC compared to that for the parental strain, the *rdxA-fdxB* dual inactivation increased the MIC twofold compared with that for Mtz-sensitive strain in which a single gene (*rdxA*) was inactivated (from 32 to 64 μg/ml). In addition, the MICs for the low-level Mtz-resistant strains (MICs, 8 μg/ml) in which a single gene (*fdxB*) was inactivated were also increased fourfold and eightfold, as shown in Table 4. These results indicate that the *fdxB* inactivation is also involved in increasing the level of Mtz resistance. It is not clear why the MIC was unchanged for an Mtz-sensitive strain with an inactivated *fdxB*

gene. However, it could be possible that the Mtz nitroreductase activities from RdxA and FrxA were much higher than that from FdxB in the Mtz-sensitive strain that contained fully functional *rdxA* and *frxA* genes, which might lead to no effect of the single *fdxB* inactivation.

Expression of the *frxA* gene cloned from *H. pylori* 26695 in *E. coli* resulted in no significant difference in the Mtz sensitivity of Mtz-resistant *E. coli* (14). However, Mtz sensitivity analysis by *frxA* inactivation of Mtz-sensitive *H. pylori* strains and *H. pylori* strains with low-level or moderate Mtz resistance showed that *frxA* inactivation conferred Mtz resistance at a level similar to that achieved by *rdxA* inactivation. In particular, nucleotide sequence analysis of the *frxA* genes from clinical isolates with moderate and high levels of Mtz resistance provided genetic evidence of the involvement of *frxA* in Mtz resistance. One moderately Mtz-resistant strain 6013 (Mtz MIC, 32 μg/ml) carried an inactivated *frxA* gene but a fully functional *rdxA* gene. Mtz sensitivity analysis of strains with inactivated *frxA* and/or *rdxA* genes showed that strain 1857 with both inactivated *frxA* and inactivated *rdxA* genes had a high level of Mtz resistance, as shown for strain 2600, in which both *frxA* and *rdxA* were inactivated. In addition, Mtz sensitivity analysis of strains with inactivated *frxA* and/or *rdxA* genes allowed us to find strain 1700, which contained a single inactivated gene (*rdxA*) and which had a high level of Mtz resistance, as was also shown for strain 2600, which had a single inactivated gene (*rdxA*). Comparative analyses of complementation of Mtz sensitivity from either an Mtz-sensitive strain to an Mtz-resistant strain, or vice versa, with inactivated or functional *frxA* and *rdxA* genes, respectively, coupled with the expression of cloned inactivated and functional *frxA* and *rdxA* genes in *E. coli*, prove that the *frxA* gene is involved in Mtz resistance. Comparison of FrxA and RdxA protein sequences from *H. pylori* ATCC 700392 showed 25% identity and 63% similarity with the absolutely conserved amino acid PW (Pro, Trp) at positions 51 and 52 of classical nitroreductases (14), indicating that the FrxA protein also possesses Mtz nitroreductase activity, as shown for the RdxA protein.

We also confirmed that the *frxA* gene from *H. pylori* strain ATCC 700392 was involved in Mtz resistance. The *frxA* gene cloned from strain ATCC 700392 [5.3-kb *Xba*I fragment in pBluescript SK(+)] was expressed in *E. coli* and converted the Mtz MICs for the cells from >320 μg/ml to 80 to 160 μg/ml. Although the difference in the MIC was not very impressive, as shown for the *frxA* gene from strain 2600 (Mtz MIC, >320 to 20- to 40 μg/ml), cloning of the *frxA* gene from strain ATCC 700392 significantly decreased the MIC. However, the MIC for strain ATCC 700392 with an inactivated *frxA* gene did not increase as much as those for strains 2617 and 2593 with inactivated *frxA* genes (for which the MICs were the same as that for strain ATCC 700392 [MIC, 8 μg/ml]), suggesting that *frxA* from strain ATCC 700392 may be partially inactivated. Indeed, 8 amino acids in the N terminus (the first 80 amino acids) of FrxA from strain ATCC 700392 were replaced by other amino acids, while none of the amino acids in the same FrxA from Mtz-sensitive strain 2600 was replaced when the sequence was compared with the FrxA sequence of Mtz-sensitive strain J99. The MIC of 8 μg/ml for strain ATCC 700392 was additional supporting evidence for the partial inactivation of the *frxA* gene. Another feature of *frxA* was the fact that a 972-bp *Eco*RI fragment and an ~2.5-kb *Sph*I-*Xba*I fragment from pGH170 (which carried the *frxA* gene from strain 2600) did not change MICs in *E. coli* (no difference in Mtz sensitivity). These results indicate that at least a 2.0-kb upstream flanking region of the *frxA* gene is required for appropriate Mtz nitroreductase expression in *E. coli*. Nucleotide sequence analysis showed that the FrxA protein followed a putative 3-hydroxyacid dehydrogenase at the carboxyl terminus with only two intervening nucleotide sequences, suggesting that the *frxA* mRNA may be cotranscribed with an upstream gene(s).

In summary, the overall finding of this study is that two genes (*fdxB* and *frxA*), in addition to the *rdxA* gene, are responsible for the Mtz resistance of *H. pylori*. The inactivation of *fdxB*, *frxA*, or *rdxA* is involved in different levels of Mtz resistance in *H. pylori*. These results lead us to hypothesize that the wide range of Mtz MICs seen for clinical *H. pylori* isolates may be due to partial and/or complete inactivation of the *fdxB*, *frxA*, and *rdxA* genes. Indeed, Mtz MICs of 32, 64, 128, and 256 μg/ml were created by inactivation of one or two of the three genes in Mtz-sensitive strains. Additionally, Mtz MICs of 8 and 16 μg/ml are also theoretically achievable by partial inactivation of the genes, as shown for strain ATCC 700392. However, the high-level Mtz resistance of some strains (e.g., strains 2600 and 1700) in which a single *frxA* or *rdxA* gene is inactivated suggests that additional Mtz resistance mechanisms exist in *H. pylori*.

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