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Sequential Inactivation of *rdxA* (HP0954) and *frxA* (HP0642) Nitroreductase Genes Causes Moderate and High-Level Metronidazole Resistance in *Helicobacter pylori*

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Helicobacter pylori **is a human-pathogenic bacterial species that is subdivided geographically, with different genotypes predominating in different parts of the world. Here we test and extend an earlier conclusion that metronidazole (Mtz) resistance is due to mutation in *rdxA* (HP0954), which encodes a nitroreductase that converts Mtz from prodrug to bactericidal agent. We found that (i) *rdxA* genes PCR amplified from 50 representative Mtz^r strains from previously unstudied populations in Asia, South Africa, Europe, and the Americas could, in each case, transform Mtz^s *H. pylori* to Mtz^r; (ii) Mtz^r mutant derivatives of a cultured Mtz^s strain resulted from mutation in *rdxA*; and (iii) transformation of Mtz^s strains with *rdxA*-null alleles usually resulted in moderate level Mtz resistance (16 μg/ml). However, resistance to higher Mtz levels was common among clinical isolates, a result that implicates at least one additional gene. Expression in *Escherichia coli* of *frxA* (HP0642; flavin oxidoreductase), an *rdxA* paralog, made this normally resistant species Mtz^s, and *frxA* inactivation enhanced Mtz resistance in *rdxA*-deficient cells but had little effect on the Mtz susceptibility of *rdxA*$^+$ cells. Strains carrying *frxA*-null and *rdxA*-null alleles could mutate to even higher resistance, a result implicating one or more additional genes in residual Mtz susceptibility and hyperresistance. We conclude that most Mtz resistance in *H. pylori* depends on *rdxA* inactivation, that mutations in *frxA* can enhance resistance, and that genes that confer Mtz resistance without *rdxA* inactivation are rare or nonexistent in *H. pylori* populations.**

Helicobacter pylori is a gram-negative microaerophilic bacterium that chronically infects human gastric epithelial cell surfaces and the overlying gastric mucin, a niche that few if any other microbes can occupy. It is carried by more than half of all people worldwide and is an important human pathogen: a major cause of peptic ulcer disease, and a contributor to other illnesses, ranging from childhood malnutrition to gastric cancer, and to increased susceptibility to other food- and waterborne pathogens (7, 8, 32, 38, 47). There is great intrinsic and public health interest in fully elucidating *H. pylori*'s metabolic pathways and how *H. pylori* maintains its redox balance during microaerobic growth. Such knowledge should help us to understand the extraordinary chronicity of *H. pylori* infection and factors that determine whether a given infection will be benign or virulent, elucidate mechanisms of drug susceptibility and resistance, and identify potential targets for new effective antimicrobial agents.

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Here we focus on mechanisms of susceptibility and resistance of *H. pylori* to metronidazole (Mtz), a synthetic nitroimidazole that is a key component of popular and affordable anti-*H. pylori* therapies worldwide and that is also widely used against various anaerobic and parasitic infections (13, 36, 45). Resistance to Mtz is common among *H. pylori* strains, with frequencies among clinical isolates ranging from 10 to >90%, depending on geographic region and patient group (17, 29, 30). Much of this is attributable to the repeated use of Mtz against other (non-*Helicobacter*) infections in regimens that are only partially inhibitory, leading to selection for resistance to *H. pylori*. This is important clinically because Mtz resistance in *H. pylori* markedly decreases the efficiency of Mtz-based eradication therapy and the cure of associated disease (15, 28).

We had traced the resistance of a Mtz^r clinical isolate to a loss-of-function mutation in *rdxA* (HP0954), a chromosomal gene for an oxygen-insensitive NADPH nitroreductase, and then identified equivalent *rdxA* mutations in 15 other Mtz^r strains from North and South America, Australia, and Europe (10, 16). Our experiments also showed that (i) mutational inactivation of *rdxA* was sufficient to cause Mtz resistance in an Mtz^s reference strain (26695); (ii) expression of *rdxA* from Mtz^s *H. pylori* strains in *Escherichia coli* rendered this normally

Mtz^r species Mtz^s; (iii) expression of a functional *rdxA*$^+$ allele on a shuttle plasmid restored Mtz susceptibility to an Mtz^r *H. pylori* strain; and (iv) new mutations in *rdxA*, not gene transfer from unrelated lineages, were often responsible for Mtz resistance in clinical isolates (16). In confirmation, *rdxA* mutations were found in 25 of 27 Mtz^r derivatives of strain SS1 obtained from infected Mtz-treated mice (22) and in 12 of 13 Mtz^r clinical isolates from France and North Africa (42) (the bases of resistance in the unusual Mtz^r strains with apparently intact *rdxA* genes were not determined). Consideration of enzyme mechanisms had indicated that Mtz activation by the RdxA nitroreductase generates nitroso- and hydroxylamine-related compounds that should be mutagenic and bactericidal (16). Mtz-induced mutation has been documented in *H. pylori* and also in *E. coli* carrying an expressed *rdxA* gene (39). Thus, recurrent exposure of resident *H. pylori* strains to Mtz, an inadvertent consequence of therapy against other common infections, may induce as well as select for Mtz resistance in this gastric pathogen.

Following our report linking *rdxA* inactivation and Mtz resistance (16), several researchers suggested that other mechanisms (presumably *rdxA* independent) might often also cause Mtz resistance (18, 27). This was based in part on observations that nominally Mtz^r clinical isolates differ in the levels of Mtz that they tolerate (resistance level, or MIC), and also fit with precedents of multiple mechanisms of drug resistance in other bacterial species (9, 33, 37). In principle, resistance might also result from (i) diminished Mtz uptake or its active export (26, 40), (ii) more efficient DNA damage repair (6, 43), or (iii) enhanced scavenging of oxygen radicals that are produced according to certain models of Mtz activation (23, 41). Of particular note are plasmid- and transposon-borne *nim* genes in certain Mtz^r strains of *Bacteroides fragilis* that promote conversion of nitroimidazoles from prodrug to harmless amino derivatives, rather than to toxic nitroso radicals, and that thus confer resistance without loss of chromosomal nitroreductase gene function (5, 46).

DNA fingerprint and sequence analyses have indicated that each *H. pylori* clinical isolate differs genetically from most other independent isolates (1, 2). Superimposed on this great general diversity, we and others have identified several subpopulations of *H. pylori* that are relatively distinct genetically, with each specific to a different geographic region or human ethnic group: one in southwest Europe (Spain); a second in East Asia; and a third in Calcutta, India (1, 21, 24, 30, 31). The strains of South and Central America seemed most closely related to southwest European (Spanish) strains, not Asian strains, as are many strains from Africa and the United States (24). Most or all strains whose Mtz resistance has been studied to date are probably of the European type; the possibility of alternative resistance genes being abundant in the gene pools of non-Western *H. pylori* strains remains to be tested.

Here we describe functional and sequence analyses that (i) establish that mutational inactivation of the *rdxA* nitroreductase gene is critically involved in primary Mtz resistance in most or all strains from South and East Asia and sub-Saharan Africa, as well as from the West; (ii) demonstrate that the resistance of Mtz^r (*rdxA*-deficient) strains can be increased by mutation in other genes, including *frxA* (flavin nitroreductase; HP0642 in reference 42); and (iii) show that *frxA* does not contribute to the normal Mtz^s phenotype of wild-type *H. pylori* strains. No evidence of determinants that bypass the need for *rdxA* inactivation in the development of clinically significant Mtz resistance was found.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *H. pylori* strains used in this study were clinical isolates from diverse parts of the world, and most have been described in references 24 and 30. The recent clinical isolates from Alaska are from Native peoples in Anchorage and other sites around the state. Strains 26695 and J99 are Mtz^s reference strains whose complete genome DNA sequences have been determined (3, 44). Each strain is from an ethnic European patient: 26695 from the United Kingdom (44), and J99 from an ethnic European in Pulaski, Tenn. (T. L. Cover, personal communication).

H. pylori strains were grown on brain heart infusion agar (Difco) supplemented with 7% horse blood, 0.4% IsoVitaleX and the antibiotics amphotericin B (8 μg/ml), trimethoprim (5 μg/ml), and vancomycin (6 μg/ml) (BHI agar). Mtz was added to this medium when needed at a concentration appropriate for the experiment, as detailed below. The plates were incubated at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). *H. pylori* transformation (electroporation) was carried out as described elsewhere (34).

E. coli DH5α was grown on Luria-Bertani medium. The small multicopy Amp^r plasmid vector pBluescript SK− (pBS) was used as a cloning vector, and cells carrying it were selected on medium with 50 μg of ampicillin/ml.

DNA methods. *H. pylori* genomic DNAs were isolated from confluent cultures grown on BHI agar using a Qiamp tissue kit (Qiagen Corporation, Chatsworth, Calif.) or the cetyltrimethylammonium bromide-phenol method (4). PCR was carried out in 20-μl volumes containing 10 ng of genomic DNA, 10 pmol of each primer, 1 U of *Taq* DNA polymerase (Promega) or high-fidelity *Taq* (Boehringer Mannheim), and 0.25 mmol of each deoxynucleoside triphosphate in standard PCR buffer. Reaction mixtures were preincubated for 2 min at 94°C and then used in 30 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 1 min per kb, with the final elongation step of 72°C for 10 min. PCR fragments were purified for sequencing by QIAquik PCR purification kit (Qiagen). Sequencing reactions were carried out using a Big Dye terminator cycle sequencing kit (PE Applied BioSystems, Foster City, Calif.), and products were run on ABI automated sequencers in the Washington University molecular microbiology core facility. The primers used are listed in Table 1.

Determination of Mtz sensitivity and resistance. Frozen *H. pylori* cultures were streaked onto Mtz-free BHI agar and incubated for 3 days; then bacterial growth was respread on fresh Mtz-free BHI agar and incubated for 1 day. The resulting young exponentially growing cells were suspended in phosphate-buffered saline; a series of 10-fold dilutions of these suspensions was then prepared, and 10 μl of each dilution was spotted on freshly prepared BHI agar containing appropriate concentrations of Mtz (variously, 0, 0.2, 0.5, 1.5, 3, 8, 16, 32, and 64 μg/ml). When the frequency of cells that formed colonies on Mtz-containing media was very low ($<10^{-6}$), estimates of viability and mutant frequency were made more accurate by spreading aliquots of cultures on the entire surface of a BHI agar petri plate, instead of spotting aliquots in small areas. A strain was considered to be susceptible to concentrations of Mtz that decreased its efficiency of colony formation at least 10-fold. This quantitative procedure was more sensitive than conventional MIC determinations, which typically estimate concentrations of antibiotic needed to block growth of denser bacterial suspensions. In particular, this procedure minimizes complications that could stem from the mutagenicity of Mtz for *H. pylori* (39), which would be exacerbated if *H. pylori* stressed by DNA-damaging agents tended to enter a hypermutable state (35).

New Mtz^r mutants. Mtz^r mutant derivatives of strain 26695 that may have induced as well as selected by Mtz (39) were obtained by spreading 10^8 bacterial cells from young cultures (as above) on BHI agar containing Mtz at 3 μg/ml. Individual colonies were streaked on BHI agar with the same Mtz concentration and then tested on BHI agar containing higher concentrations of Mtz (8, 16, 32, and 64 μg/ml).

Engineered *H. pylori* strains. (i) *rdxA* mutants. The *rdxA*::*cam* allele, which contains a *cam* cassette in the *rdxA* gene (16), was moved to the chromosome of *H. pylori* strains by DNA transformation and selection on BHI agar with 15 μg of chloramphenicol (Cam)/ml. Transformants were checked to verify that they had resulted from allelic replacement by PCR using primers *rdxA*-F and *rdxA*-R, which generates products of 2 kb in cases of replacement and 886 bp in cases of retention of the original *rdxA* allele (Table 1).

A deletion of nearly all of *rdxA* (601 bp of the 630-bp open reading frame) (*rdxA*Δ601) was engineered as follows. A 1,343-bp PCR product was generated by amplification of strain 26695 genomic DNA with oligonucleotide primers specific for genes that flank *rdxA* (primers *rdxA*-F1 and *rdxA*-R1) and cloned into the *Eco*RV site of a pBS plasmid vector. A second PCR was carried out using the *rdxA* plasmid clone with outward facing primers specific for sites near the 5′ and 3′ ends of *rdxA* (primers *rdxA*-F2 and *rdxA*-R2), and the linear products were ligated and recovered as circular plasmids in *E. coli* DH5α. DNA containing this *rdxA*Δ601 allele was introduced into Mtz^s *H. pylori* strains by electroporation, with selection for Mtz resistance on BHI agar with 3 or 8 μg of Mtz/ml, with equivalent results. Mtz^r transformants were tested by PCR with primers *rdxA*-F1 and *rdxA*-R1 to see if they had resulted from the desired allelic exchange; a product of 742 bp, rather than 1,343 bp, indicated replacement.

A 111-bp in-frame deletion in *rdxA* (*rdxA*Δ111) was engineered, essentially as with *rdxA*Δ601, using outward-facing primers containing *Xba*I sites near their 5′ ends (*rdxA*-F3 and *rdxA*-R3). *Xba*I digestion of the linear PCR product, ligation,

TABLE 1. Primers used

Primer	Sequence[a]	Remark
Inward facing		
rdxA-F	5′-GCAGGAGCATCAGATAGTTCT-3′	886-bp product
rdxA-R	5′-GGGATTTTATTGTATGCTACAA-3′	
rdxA-F1	5′-CGGACTCATGGAATTGCTCCAT-3′	1,343-bp product
rdxA-R1	5′-GGCAAATCATAGGCATTATGGTG-3′	
Outward facing		
rdxA-F2	5′-ATGGAAAAATTTCATTGATTTTCC-3′	601-bp deletion
rdxA-R2	5′-GCGATTACTTGGTTGTGATTAA-3′	
rdxA-F3	5′-GTCATCTAGACCTGGCGATTTCAGCGATTTCT-3′	111-bp deletion
rdxA-R3	5′-GTCATCTAGAAGCGCTTCAGCGTTAATGGTGGT-3′	
Inward facing		
frxA-F1	5′-GCGCTTCAAAGCTTGGGTTACCA-3′	1,383-bp product
frxA-R1	5′-GCCTTCAATGTTGCGCTCTTTGT-3′	
Outward facing		
frxA-F2	5′-GGCGTGTCGGTAATGGCTTGTT-3′	523-bp deletion
frxA-R2	5′-GGTGCTGTAAAGCAACCACTTGT-3′	
Inward facing		
1508-F1	5′-CGGATCCAGCCACTCTAGCCA-3′	1,980-bp product
1508-R1	5′-GCACGCAAGACATTCGTTGCG-3′	
Outward facing		
1508-F2	5′-CCAAGGCTAGTGGTGATGA-3′	1,108-bp deletion
1508-R2	5′-GCACCAAGTGAGGATTGAAG-3′	
Inward facing		
588-F1	5′-GCGATTAGAGCCGTGATCTTC-3′	976-bp product
588-R1	5′-CGCATCACATCAGCGATCACT-3′	
Outward facing		
588-F2	5′-CATGCTCTTAGAAGAGACTA-3′	537-bp deletion
588-R2	5′-CCTTACACCTGTCTTCATTC-3′	
Inward facing		
*cagA*2143F	5′-GCAGAAGAAACGCTAAAAGCCCTT-3′	1,370-bp product
*cagA*3512R	5′-CTTCCCACATTATGCGCAACTAT-3′	
Outward facing		
*cagA*2900F	5′-CGATTGATGATCTCGGCGGACCTTT-3′	226-bp deletion
*cagA*2673R	5′-GGGTTCGTTTTCGAGTCCATTATTGT-3′	

[a] Underline indicates *Xba*I site.

recovery in DH5α, transformation into *H. pylori* with selection for Mtz resistance, and PCR verification were carried out as described above.

(ii) *frxA* gene cloning and construction of *frxA*::*cam* and *frxA*::*kan* insertion/deletion mutants. The *frxA* flavin nitroreductase gene segment (HP0642 in the strain 26695 genome [44]) was PCR amplified from *H. pylori* genomic DNAs using primers *frxA*-F1 and *frxA*-R1 or, in some experiments, *frxA*-F and *frxA*-R. The amplified DNAs were cloned into the *Eco*RV site of plasmid pBS and recovered after transformation of *E. coli* DH5α.

A marked 523-bp deletion in *frxA* from strain 26695 was generated by PCR using outward-facing *frxA* primers *frxA*-F2 and *frxA*-R2, followed by ligation with a *cam* cassette (as in reference 16) or a *kan* cassette (25). Mutant plasmids were recovered in DH5α using selection for Cam^r (20 μg/ml) or Kan^r (20 μg/ml). The *frxA*::*cam* and *frxA*::*kan* alleles were introduced into *H. pylori* by transformation and selection for Cam^r (15 μg/ml) or Kan^r (20 μg/ml), as appropriate; the structures of transformants were verified by PCR with primers *frxA*-F1 and *frxA*-R1, as above.

(iii) HP1508::*cam* insertion/deletion mutation. The HP1508 gene, which encodes a ferredoxin-like protein of unknown function, was PCR amplified from strain 26695 DNA, using primers 1508-F1 and 1508-R1, and similarly cloned into pBS. A marked 1,108-bp internal deletion was generated using PCR with outward-facing primers 1508-F2 and 1508-R2, followed by ligation with a *cam* cassette, as above. The structures of Cam^r transformants were verified by PCR with primers 1508-F1 and 1508-R1.

(iv) *oorD*::*cam* insertion/deletion mutation. The *oorD* (HP0588) gene, which encodes the ferridoxin component of the multisubunit oxoglutarate oxidoreductase enzyme (20), was PCR amplified from strain 26695 DNA, using primers 588-F1 and 588-R1, and cloned into pBS. A marked 537-bp internal deletion was generated using PCR with outward facing primers 588-F2 and 588-R2, followed by ligation with a *cam* cassette, as above. The structure of the one *H. pylori* transformant obtained (see Results) was verified by PCR with primers 588-F1 and 588-R1.

(v) Addition of a functional *rdxA* gene to the *H. pylori* genome. A functional *rdxA* gene, PCR amplified from strain 26695 with primers *rdxA*-F1 and *rdxA*-R1, and the *cam* cassette were cloned into the *Eco*RV and *Sma*I sites, respectively, of plasmid pBS (each gene transcribed toward the other). In parallel, a 1.37-kb segment of *cagA*, PCR amplified from strain NCTC11637 using primers *cagA*2143F and *cagA*3512R, was cloned into the *Sma*I site of pBS. The resulting plasmid was linearized by PCR with outward-facing primers *cagA*2673R and *cagA*2900F, which are specific to sites 531 and 613 bp from the two ends of the cloned *cagA* DNA fragment. The *rdxA-cam* segment was then PCR amplified from the pBS-*rdxA-cam* construct, using pBS-specific primers M13F and M13R, and cloned between the 531- and 613-bp fragments of the *cagA* gene. An isolate in which *rdxA* was oriented in the same direction as *cagA* was used to transform NCTC11637 to Cam^r. The structures of resultant transformants were verified by PCR using primers *cagA*2143F and *cagA*3512R.

RESULTS

Intrinsic Mtz susceptibility or resistance of *H. pylori* reference strains and clinical isolates. To determine the lowest concentrations of Mtz that permitted survival of reference strains 26695 and J99, young cultures were diluted serially, aliquots of dilutions were spotted on Mtz-containing BHI agar, and numbers of colonies formed at appropriate dilutions were determined. Strain 26695 exhibited an efficiency of plating (EOP) of ~1 on BHI agar with up to 1.5 μg of Mtz/ml and $\sim 10^{-4}$ on BHI agar with 3 or 8 μg of Mtz/ml (phenotype designated 1.5R 3S). Reference strain J99 was somewhat more susceptible, exhibiting EOPs of ~1 and 10^{-3} on BHI agar with 1 and 1.5 μg of Mtz/ml, respectively (phenotype designated 1R 1.5S) and 10^{-4} on BHI agar with 3 or 8 μg of Mtz/ml (Fig. 1).

H. pylori strains from patients from five continents, chosen to represent much of the diversity of this pathogen worldwide, were divided into two groups based on a first-pass test of susceptibility or resistance to Mtz, defined as inability or ability

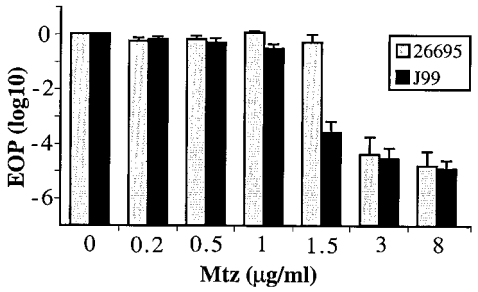

FIG. 1. Profiles of intrinsic susceptibility and resistance to Mtz of reference strains 26695 and J99. Young exponentially growing cultures were diluted, aliquots were spotted on BHI agar with indicated concentrations of Mtz, and surviving colonies were counted from appropriate dilutions, as detailed in Materials and Methods. Presented are the average and range of results with two single colony isolates (cultures) of each strain, with assays repeated three times with each culture.

to grow on BHI agar containing Mtz at 8 μg/ml (a concentration generally used clinically as a threshold for significant resistance). The levels of Mtz just sufficient to kill representative strains from each group were then determined more precisely, as with the reference strains above. The Mtz^s strains included 20 from Japan, a society in which Mtz use is rare and hence in which *H. pylori* strains should have had little inadvertent exposure to this drug, as well as strains from societies in which Mtz use is common and in which more than half of strains are resistant (India, Peru, and South Africa). Forty-eight of these 61 strains tested were like strain 26695 in phenotype (1.5R 3S), and another seven were like strain J99 (1R 1.5S) (Table 2). Two were more susceptible (0.5R 1.0S), and four were marginally more resistant (3R 8S).

In equivalent characterizations of 55 representative Mtz^r clinical isolates, nearly 40% were resistant to just 16 μg/ml (16R 32S; 21 of 55 strains), another 40% were resistant to just 32 μg/ml (32R 64S; 22 of 55 strains), and 16% exhibited higher resistance (64R; 9 strains). Just 3 of the 55 strains exhibited lower resistance (8R 16S) (Table 3).

New Mtz^r mutants generated in culture. To test our inference that Mtz resistance generally involves decreased *rdxA* function, new mutant Mtz^r derivatives of reference strain 26695 were selected on BHI agar containing just 3 μg/ml, the lowest concentration of Mtz that allowed Mtz^r mutants to emerge cleanly from background growth. Such mutants were obtained at frequencies of about 10^{-4} in cultures from different single-colony isolates, as noted above (Fig. 1).

Only 13 of these 149 mutants selected for resistance to at least 3 μg/ml were susceptible to Mtz at 8 μg/ml (phenotype designated 3R 8S). Each of the other 137 mutants was resistant to at least 8 μg/ml. Of these, 39 were unable to grow on BHI agar with 16 μg/ml (8R 16S phenotype), 97 grew well with 16 but not 32 μg/ml (16R 32S phenotype), and one exceptional mutant (mutant 0161) grew well with 32 μg of Mtz/ml (32R 64S phenotype). The differences in distributions of levels of Mtz resistance among clinical isolates versus newly arisen mutants (≥32R phenotype in 56% of clinical isolates versus <1% of newly arisen mutants; conversely, 8R 16S phenotype in only 10% of clinical isolates versus 29% of new mutants) suggested both that *H. pylori* is often exposed to relatively high concentrations of Mtz during human infection and that high-level resistance (≥32 μg/ml) might arise in several steps.

TABLE 2. Distribution of MICs among Mtz^s *H. pylori* isolates[a]

Geographic region	No. of strains	Mtz phenotype				
		0.2R 0.5S	0.5R 1S	1R 1.5S	1.5R 3S	3R 8S
Japan	20	0	0	0	20	0
China	9[a]	0	1	0	6	2
India	4	0	0	2	1	1
South Africa	8	0	1	1	5	1
Alaska	5	0	0	2	3	0
Spain	10	0	0	1	9	0
Peru	5	0	0	1	4	0
Total	61	0	2	7	48	4

[a] Five strains from Shenyang; four strains (including one with 0.5R 1S phenotype) from Hong Kong.

TABLE 3. Distribution of MICs among Mtz^r *H. pylori* isolates

Geographic region	No. of strains	Mtz phenotype			
		8R 16S	16R 32S	32R 64S	64R
China[a]	6	2	3	1	0
India	20	0	8	6	6
South Africa	7	0	2	2	3
Alaska	11	0	5	6	0
Peru	11	1	3	7	0
Total	55	3	21	22	9

[a] Each of the Chinese strains is from Shenyang.

Nature of newly arisen Mtz^r mutants. Eight low-level Mtz^r mutants (3R 8S) were characterized by sequencing. Three contained mutations in or immediately upstream of *rdxA*, whereas the other five did not (Table 4, group A); the mutations that caused the weak Mtz resistance of these latter five isolates have not been identified. The *rdxA* genes of four independent Mtz^r mutants with the more common higher-level Mtz resistance were also sequenced (two 8R 16S, one 16R 32S, and one 32R 64S). Simple point mutations in *rdxA* were found in each case (Table 4, group B), as expected (16).

The possibility of clinically significant resistance to Mtz arising by stepwise accumulation of mutations in loci other than *rdxA* (without *rdxA* inactivation) was tested using three of the weakly Mtz^r mutants (3R 8S phenotype), in which resistance was due to mutation outside of *rdxA*. Five of six derivatives selected on BHI agar with Mtz at 8 μg/ml had a 16R 32S phenotype, and the sixth had a 32R 64S phenotype. Each of these six contained a new point mutation, either in the *rdxA* open reading frame (five cases) or in the Shine-Dalgarno sequence just upstream of *rdxA* (one case) (Table 4, group C). These results support the conclusion that resistance to the more clinically significant levels of Mtz usually involves *rdxA* inactivation.

Loss-of-function mutations in *rdxA* associated with Mtz^r worldwide. The idea that *rdxA* inactivation is critically involved in most or all clinically significant cases of Mtz resistance was tested against an alternative possibility, that Mtz resistance in certain geographic regions might often result from auxiliary (e.g., plasmid or transposon) resistance genes that are uncommon in Western *H. pylori* strains and that bypass the need for *rdxA* inactivation. This entailed PCR amplification of a segment containing *rdxA* from Mtz^r strains from various representative populations (12 Chinese, 12 Indian, 11 Alaska Native, 9 Peru Native, and 6 South African), electroporation of the Mtz^s strain 26695 with these PCR-amplified *rdxA* DNAs, and quantitation of the yield of Mtz^r transformants on BHI agar with 8 μg of Mtz/ml (Fig. 2). Mtz^r transformants were

TABLE 4. Spontaneous mutation in *rdxA* and amino acid substitutions in *H. pylori* 26695

Mutant	Mtz phenotype	Substitution	
		Nucleotide	Amino acid[a]
Group A, 3R 8S			
202	8R 16S	CGC→TGC	Arg(16)→Cys
407	3R 8S	CCA→CTA	Pro(96)→Leu
301	3R 8S	A→G	37 bp upstream from start codon (ATG)
201	3R 8S		
116	3R 8S		
515	3R 8S		
619	3R 8S		
251	3R 8S		
Group B, ≥8R			
501	8R 16S	GCA→GAA	Ala(67)→Glu
621	8R 16S	GAT→GGT	Asp(23)→Gly
111	16R 32S	GCT→GTT	Ala(80)→Val
161	32R 64S	GAG→TAG	Glu(75)→stop
Group C, 3R 8S→8R			
1001	32R 64S	CGC→AGC	Arg(16)→Ser
1161	16R 32S	CGC→CAC	Arg(16)→His
2002	16R 32S	CTA→-TA	Frameshift from Leu(42)
5151	16R 32S	GAG→TAG	Glu(75)→stop
1162	16R 32S	GAG→TAG	Glu(175)→stop
2001	16R 32S	AAGGAA→AAAGAA	Shine-Dalgarno ribosome-binding site

[a] Position of the amino acid substitution is given in parentheses.

obtained with frequencies of about 10^{-2}, using *rdxA* DNA amplified from each of the 50 Mtz^r clinical isolates tested and also from strain SS1*rdxA*Δ111, as a positive control. This frequency was 100-fold higher than the yield of Mtz^r colonies obtained with PCR products from each of six control Mtz^s strains ($\sim 10^{-4}$) (two Alaska Native, two South African, and two Indian), indicating that each of the 50 Mtz^r strains contained mutant alleles of *rdxA*. It is also noteworthy that each of the 50 *rdxA* mutant PCR products was of the size expected (~890 bp), indicating that each contained a point mutation, not an insertion or deletion, in *rdxA*. These results showed that *rdxA* inactivation is critically involved in most cases of Mtz resistance worldwide and ruled out a model (14) in which changes in regulatory genes that affect expression of *rdxA* and/or other reductase genes would be responsible for most Mtz resistance in clinical isolates.

RdxA as principal determinant of Mtz susceptibility of most wild-type strains. Initial tests had shown that transformants of the Mtz^s strain 26695 obtained using an *rdxA*::*cam* cassette and selected for Cam^r were Mtz^r in phenotype (16). In the present, more quantitative tests, this *rdxA*::*cam* mutant strain exhibited a 16R 32S phenotype, as did most newly arisen Mtz^r mutants. Equivalent 16R 32S phenotypes were exhibited by derivatives of strain 26695 containing unmarked *rdxA* deletion alleles (*rdxA*Δ601 or *rdxA*Δ111) (Fig. 3), in each case selected after DNA transformation and selection for resistance to just 3 μg of Mtz/ml. These results showed that *rdxA* encodes the only nitroreductase sufficiently active to confer an Mtz^s phenotype on this reference strain.

The generality of these results was tested by transformation using the *rdxA*::*cam* allele and a sampling of strains with normal Mtz^s phenotypes (1.0R 1.5S or 1.5R 3S) (i) from a society in which Mtz use is uncommon and Mtz resistance is rare (29) and (ii) from societies in which Mtz use is more common and many (in some countries, most) strains are Mtz^r. Inactivation of *rdxA* led to an Mtz^r phenotype in 26 of the 28 strains tested

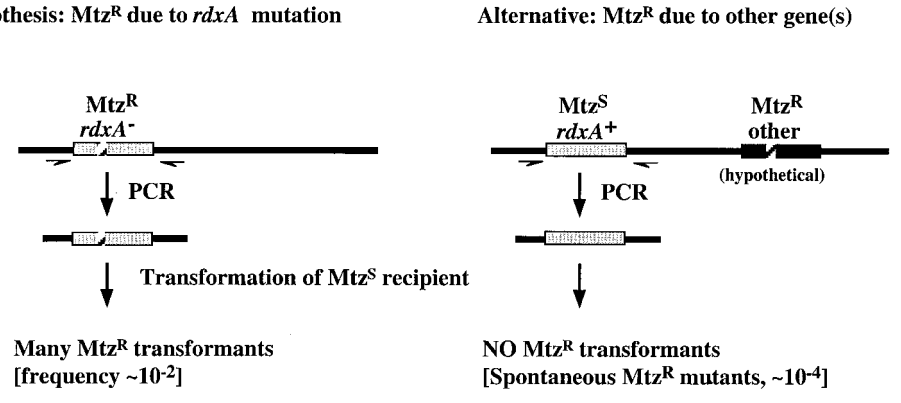

FIG. 2. DNA transformation strategy for testing involvement of *rdxA* gene mutation in Mtz^r clinical isolates.

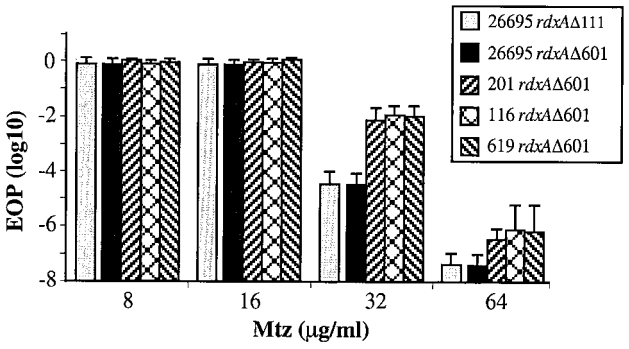

FIG. 3. Mtz resistance profiles of *rdxA*Δ111 or *rdxA*Δ601 deletion mutant transformants of strain 26695 and of *rdxA*Δ601-containing transformants of three 26695 derivatives that had exhibited an unusual 3R 8S phenotype that was not attributable to mutation in *rdxA* (201, 116, and 619 [Table 4]). Transformants containing the *rdxA*Δ111 and *rdxA*Δ601 alleles were selected using BHI agar with 8 μg of Mtz/ml. Presented are the average and range of results with three single colony isolates (cultures) of each strain, with each culture assayed two times.

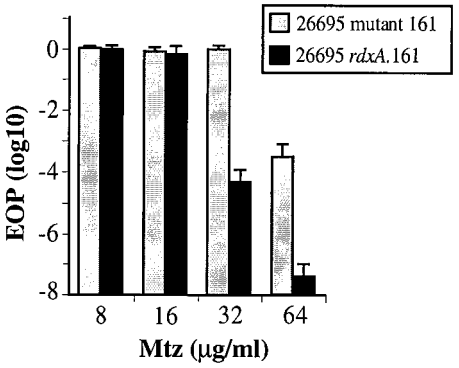

FIG. 4. Mtz resistance profiles of the unusual highly resistant mutant of strain 26695 (designated 26695 mutant 161 [Table 4]) and also a 26695 derivative that contains only the *rdxA* gene from this mutant (designated *rdxA*.161). The *rdxA*.161 transformant was selected on BHI agar with 8 μg of Mtz/ml. The presence of the expected GAG-to-TAG change in *rdxA* (Table 4) was verified by DNA sequencing. Presented are the average and range of results with five single colony isolates (cultures) from each strain, with each culture assayed once.

(8 from Japan, 5 of 6 from South Africa, 5 of 6 from Hong Kong, 3 each from Peru and Spain, and 2 from India). *rdxA* inactivation led to a 16R 32S phenotype in 24 of these strains and an 8R 16S (nearly as resistant) phenotype in the other two. Further analysis of the other two (most interesting) exceptions, implicating RdxA and one other expressed nitroreductase in their unusual susceptibility to Mtz, is presented below.

Mutations in other genes can affect the level of Mtz resistance. Three sets of results established that the level of resistance of a typical Mtz^r (*rdxA*-deficient) *H. pylori* strain can be affected by other genetic determinants. First, introduction of the *rdxA*Δ601 deletion into three derivatives of strain 26695 with weak Mtz^r phenotypes (3R 8S) that were ascribed to unknown sequence changes outside *rdxA* (see above) resulted in 16R 32S phenotypes in each case. However, the EOP of these transformants on BHI agar with 32 μg of Mtz/ml was reproducibly $\sim 10^{-2}$—that is, several hundred-fold higher than that of control *rdxA*Δ601 transformants of 26695 wild type, selected in parallel (Fig. 3). This suggests that the mutations responsible for the 3R 8S phenotype may have affected process(es) distinct from those controlled by RdxA nitroreductase itself.

Second, mutant derivatives of 26695 carrying an *rdxA* deletion (16R 32S phenotype) that could grow on BHI agar with 32 or 64 μg of Mtz/ml were selected. These mutants were obtained at frequencies of about 10^{-4} and 10^{-7} (selection at 32 and 64 μg of Mtz/ml, respectively), using strains carrying the *rdxA*Δ111 or *rdxA*Δ601 deletion allele. In contrast, such hyperresistant (64R) mutants were not obtained from 26695 wild type (*rdxA*$^+$) (frequency, $<10^{-9}$). Thus, the enhanced resistance that these additional mutations confer depended on *rdxA* inactivation: these mutations did not bypass the need to mutate *rdxA* in order to develop a resistant phenotype.

Third, more than half of the 55 Mtz^r clinical isolates that we screened had 32R or 64R phenotypes (Table 3). Transformants of strain 26695 made with *rdxA* genes from two hyperresistant Indian strains and selected on BHI agar with just 8 μg of Mtz/ml were examined carefully. Each exhibited a 16R 32S (moderate resistance) phenotype, not the 64R (hyperresistance) phenotype of their DNA donor parents. Similarly, transformants made with the *rdxA* gene from a mutant derivative of 26695 that was unusual in exhibiting a 32R 64S phenotype (161 [Table 4, group B]) were also only 16R 32S in phenotype (Fig. 4). Thus, clinical isolates and laboratory mutants with very high level resistance must have contained an additional mutation that enhanced the moderate resistance conferred by simple point mutations in *rdxA*.

Flavin nitroreductase (*frxA* gene product) contributes to residual Mtz susceptibility of *rdxA* mutant strains. Theoretical considerations had suggested that *frxA* [HP0642, encoding NAD(P)H-flavin oxidoreductase; an *rdxA* paralog] might contribute to Mtz susceptibility in *H. pylori* (16). Although one *frxA* gene clone did not make *E. coli* susceptible to Mtz (16), further studies identified an *frxA*-containing cosmid clone from an *H. pylori* strain with a high-level Mtz^r phenotype (32R) (strain 439 in reference 16) that increased the yield of Mtz^r *H. pylori* transformant colonies when mixed with *rdxA* mutant DNA but did not transform Mtz^s *H. pylori* to Mtz^r when used alone. Given these various results, we elected to reexamine the possibility of a role for *frxA* in Mtz susceptibility and resistance. First, we sought to again PCR amplify and clone *frxA*-containing DNA segments from several different Mtz^s *H. pylori* strains, but using a high-fidelity *Taq* polymerase formulation to minimize mutation during PCR. Four of ten independent *frxA*-containing pBS plasmid clones that were recovered in *E. coli* DH5α (two from 26695; one each from SS1 and HP500) resulted in susceptibility to Mtz. In quantitative determinations using *frxA* clones from 26695, the EOPs were about 0.01 and 0.001 on L agar with 1 and 2.5 μg of Mtz/ml, respectively, whereas the parental *E. coli* strain (lacking *frxA*) exhibited an EOP of 1 on L agar with 50 μg of Mtz/ml. It was also noted that *E. coli* carrying cloned functional *frxA* genes tended to make small colonies on Mtz-free L agar. This result suggested that the poor yield of frxA-containing clones that rendered *E. coli* Mtz^s (only 4 of 10) might be due to some toxicity of *frxA* when hyperexpressed and that the initial lack of Mtz susceptibility associated with *frxA* cloning (16) was a spurious result, perhaps reflecting mutation during PCR or cloning and unwitting selection of a healthy (*frxA* mutant) transformant colony.

In a second test, *frxA* was sequenced from three strains that were resistant to high levels of Mtz (≥32 μg/ml). An ATG-to-ATA change was found in the start codon of *frxA* in a highly resistant mutant derivative of an *rdxA*-deficient transformant of strain 26695 (64R instead of 16R 32S in phenotype); similarly, −1 frameshift mutations were found in poly(A) tracts at nucleotide positions 48 and 310 of *frxA* in two highly resistant (32R) derivatives of SS1 that also carried an *rdxA*-null muta-

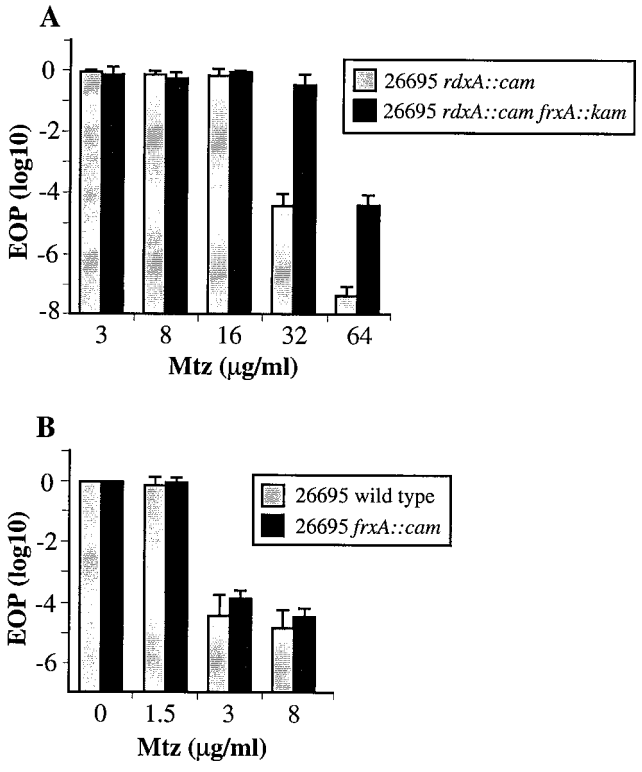

FIG. 5. Effect of *frxA* inactivation on Mtz susceptibility and resistance depends on whether *rdxA* is functional or not. (A) Profile of Mtz susceptibility of an *frxA*-null mutant of an *rdxA*::*cam* strain and of its *frxA*$^+$ parent; (B) profile of Mtz susceptibility of an *frxA*-null mutant derivative of 26695 (*rdxA*$^+$) and of its wild-type parent. Presented are the average and range of results with three single colony isolate cultures of each strain, with each culture assayed two times.

tion. In accord with this are recent descriptions of *rdxA* and *frxA* from the type strain of *H. pylori*, NCTC11637, which also exhibits a 32R phenotype: it contains a mini-IS*605* insertion and adjacent deletion in *rdxA* (10) and also a frameshift mutation in *frxA* (D. H. Kwon et al., GenBank accession no. AF225923).

In a third test, *frxA*::*kan* transformant derivatives of *rdxA*::*cam* (16R phenotype) derivatives of three normal Mtz^s *H. pylori* strains were constructed: 26695, HUP-B57, and HK192, from England, Spain, and Hong Kong, respectively. Each *rdxA frxA* double mutant exhibited a 32R 64S phenotype (Fig. 5A). In contrast, *frxA* inactivation in strains with functional *rdxA*$^+$ genes had little if any effect on their intrinsic susceptibility to Mtz (1.5R 3S phenotype) (Fig. 5B). Similarly, inactivation of *frxA* in each of five other new Mtz^s clinical isolates did not affect their intrinsic Mtz susceptibility (phenotypes 1.5R 3S in two Hong Kong and two Spanish strains; 1R 1.5S in one Peruvian strain). In accord with this, incorporation of a functional *rdxA* gene into the chromosome (as a *rdxA*$^+$ *cam* cassette flanked by segments of the *cagA* gene) of the *rdxA* and *frxA* mutant type strain NCTC11637 caused a change in its phenotype from 32R 64S to 0.5R 1S. In sum, these studies showed that loss of *frxA* function contributes significantly to Mtz resistance in *H. pylori*, but generally only if *rdxA* is also mutant. The near absence of effect of *frxA* inactivation on the Mtz^s phenotype of *rdxA*$^+$ strains is in agreement with findings that *rdxA* inactivation is generally sufficient to cause an Mtz^r phenotype.

The two unusual clinical isolates that had remained Mtz^s after *rdxA* inactivation (described above) were then studied further. Transformation using *frxA*::*kan* DNA of derivatives of these two strains that already carried *rdxA*::*cam* alleles resulted in a 32R 64S Mtz^r phenotype in each case, whereas equivalent transformation of the original *rdxA*$^+$ parental strains with the *frxA*::*kan* allele resulted in retention of Mtz^s phenotypes. Thus, these two clinical isolates were unusual in requiring inactivation of both *frxA* and *rdxA* to achieve a clinically significant Mtz^r phenotype. Although the basis of their unusual FrxA activity (e.g., high expression of the *frxA* gene versus unusually high specific activity of the FrxA product) is currently under study, these two exceptions also reinforce the sense that mutation in *rdxA* and *frxA* are each important in the development of resistance to levels of Mtz higher than can be achieved by *rdxA* inactivation alone.

Other possible contributors to resistance. As noted above, the 26695 *rdxA frxA* double mutant had a 32R 64S phenotype. Derivatives with higher resistance (64 instead of 32 μg of Mtz/ml) were recovered at a frequency of $\sim 10^{-4}$, in contrast to $\sim 10^{-7}$ in the case of *frxA*$^+$ *rdxA*-deficient strains (Fig. 5A). This indicated that resistance can be enhanced by mutation in at least one additional locus.

HP1508, which encodes a putative ferredoxin-like protein of unknown function, was tested for possible effects on Mtz susceptibility or resistance after transformation with a HP1508::*cam* insertion allele. Cam^r transformant derivatives of strain 26695 made in the *rdxA*$^+$ background had a normal 1.5R 3S phenotype; those in a *rdxA*Δ111 background had a normal 16R 32S phenotype; and those in an *rdxA*Δ111 *frxA*::*kan* background had a normal 32R 64S phenotype. The generality of this result was tested by transforming the HP1508::*cam* insertion allele into six other Mtz-susceptible clinical isolates (two from Hong Kong; one each from India, Peru, Spain, and South Africa). No effect of HP1508 inactivation on intrinsic Mtz susceptibility was detected in any of these six strains.

Equivalent tests were also attempted with *cam* insertion alleles of HP0558, which encodes the ferridoxin component of the multisubunit oxoglutarate oxidoreductase, an enzyme considered to be essential for viability (20). One Cam^r colony was obtained in several attempts to transform strain 26695 with HP0558::*cam* insertion mutant DNA under conditions that normally yield hundreds or thousands of transformants. PCR tests confirmed that this one exceptional Cam^r colony contained a replacement of the wild-type allele with the HP0588::*cam* insertion allele (data not shown). However, attempts to transform 26695 wild type with genomic DNA from this HP0588::*cam* sibling strain were also unsuccessful. Assuming that this gene is normally essential for viability (20), the one exceptional transformant obtained is inferred to contain a bypass-suppressor mutation at another locus, sufficient to allow survival without HP0588 function. In terms of the present Mtz resistance studies, it is noteworthy that the 26695 HP0588::*cam* strain exhibited a normal Mtz^s phenotype (1.5 3S) and that transformation of the *rdxA*Δ111 allele into this strain resulted in a normal 16R 32S Mtz^r phenotype. Thus, if it is assumed that the putative suppressor mutation does not affect Mtz reduction, these results would suggest that the HP0588-encoded ferredoxin does not contribute to the Mtz susceptibility of wild-type *H. pylori* or to the residual Mtz susceptibility of *rdxA* mutants.

DISCUSSION

We have studied mechanisms of susceptibility and resistance to Mtz in *H. pylori* strains from diverse parts of the world, motivated in part by recent findings that different *H. pylori*

genotypes predominate in East Asia, South Asia, and Europe, and that Latin American and African and many U.S. strains tend to be most closely related to those of Europe, not Asia (24, 30, 31). Here we present mutational, gene cloning, and sequence analyses that confirm and extend our initial conclusions (10, 16) in establishing (i) that the lethality of Mtz to wild-type *H. pylori* depends primarily on the activity of an oxygen-insensitive NADPH nitroreductase encoded by the *rdxA* (HP0954) gene, which mediates conversion of Mtz from harmless prodrug to toxic and mutagenic product (16, 39); and (ii) that Mtz resistance generally results, at least in part, from mutations that inactivate *rdxA*.

In the present studies of East Asian (China and Japan), South Asian (Calcutta), South African, and Alaska Native strains, as well as Western (Spain and Amerindian Peruvian) strains, we found (i) that a functional *rdxA* nitroreductase gene is primarily responsible for the high susceptibility to Mtz of most or all wild-type *H. pylori* strains; (ii) that clinically significant Mtz resistance generally requires mutation in *rdxA*; and (iii) that the level of Mtz resistance that a strain exhibits can be further enhanced by additional changes elsewhere in its genome, but only if it is already mutant in *rdxA*. With only a few possible exceptions (discussed below), no evidence of auxiliary resistance genes that confer clinically significant Mtz resistance without *rdxA* inactivation was found in any population. This is noteworthy, because many of the strains examined came from societies in which *H. pylori* infection and Mtz usage are frequent—conditions that would have favored the spread of any plasmid- or transposon-borne auxiliary resistance determinants.

That additional genes might also be important is emphasized by the finding that more than half of Mtz^r clinical isolates were resistant to levels of Mtz higher than can be obtained by inactivation of *rdxA* alone (Table 3). Further analyses indicated that this enhanced resistance can occur stepwise, by mutation in at least two other loci in strains already mutant in *rdxA*. First, mutational inactivation of *frxA* (HP0642), an *rdxA* homolog that encodes a related reductase (24% amino acid sequence identity), increased the resistance of *rdxA*-deficient *H. pylori* from 16 to 32 μg/ml. However, *frxA* inactivation, by itself, had little effect on the intrinsic Mtz susceptibility of Mtz^s strains. This is in accord with evidence that *rdxA* inactivation is sufficient to render Mtz^s strains Mtz^r. In this context, our finding that cloned *frxA*$^+$ genes from each of several Mtz^s *H. pylori* strains made *E. coli* highly susceptible to Mtz suggests that synthesis or activity of the FrxA reductase may be downregulated in *H. pylori*. In accord with this view, we found two unusual clinical isolates that became Mtz^r only if *rdxA* and *frxA* were each inactivated. In parallel studies, we have also found that the special mouse-adapted strain SS1 also requires inactivation of both *frxA* and *rdxA* to achieve an Mtz^r phenotype and that *frxA* mRNA levels in this strain are higher than in reference strains (J. Y. Jeong and D. E. Berg, unpublished data). We have begun to search for the putative regulatory gene(s) and/or site(s) that may be mutant in these strains and to test the possibility that unusually high FrxA activity may contribute to bacterial fitness in certain hosts.

Even higher-level resistance (64R phenotype) is common among clinical isolates and is readily obtained in culture, starting with an *rdxA frxA* double-mutant strain. Hence, at least one other gene must be involved in residual Mtz susceptibility and the emergence of hyperresistance. The involvement of other reductase enzymes in susceptibility is suggested by our finding that Mtz can be mutagenic even for hyperresistant *H. pylori* strains, since Mtz-promoted mutagenesis reflects enzymatic reduction of Mtz (39). The gene responsible for this third incremental component of hyperresistance has not yet been defined.

The multiplicity of metabolic and housekeeping functions that potentially can affect Mtz susceptibility and resistance is further illustrated by our finding that five of the eight derivatives of strain 26695, selected for very slight decreases in susceptibility to Mtz (3R instead of 1.5R phenotype), had resulted from mutation outside of *rdxA*. One explanation invokes polar mutations in upstream sequences that simply decrease *rdxA* expression. This explanation seems unlikely, however, since the level of Mtz resistance achieved after transformation of these mutants with an *rdxA* deletion allele was slightly but reproducibly higher than in their isogenic parent (Fig. 3). This implies that the 3R 8S and *rdxA* mutations affect quite different processes or pathways. The mutations are also unlikely to be in *frxA*: their enhancement of Mtz resistance in strain 26695 wild type, although slight, was greater than that conferred by an *frxA*-null allele, whereas they had less effect on Mtz resistance than the *frxA*-null allele in the *rdxA*-null background. Given the mutagenic and DNA-damaging effects of products of Mtz activation (39) and the dramatic increase in Mtz susceptibility caused by *recA* gene inactivation (43), these subtle mutant phenotypes might be ascribed to changes in genes affecting DNA replication or repair (6, 43), or equally to changes in efficiency of Mtz uptake (26) or efficiency of physiologic adaptation to growth with Mtz (19).

Also meriting further study are a few exceptional Mtz^r strains that were reported by others to contain normal *rdxA* sequences: 2 of 27 Mtz^r variants recovered from mice infected with strain SS1 and treated subtherapeutically with Mtz (22), and 1 of 13 Mtz^r strains from France and North Africa (42). It should now be possible to learn if any of these unusual mutants have decreased RdxA synthesis or activity, or if any of them result from an alternative, but still rare, mechanism for Mtz resistance that bypasses the need for *rdxA* inactivation.

The distribution of various levels of Mtz resistance among clinical isolates differs markedly from that obtained by one-step forward mutation to Mtz^r in culture. We propose that this distribution reflects a complex dynamic, including (i) the mutagenic effects of Mtz activation; (ii) the intensity of selection for Mtz^r phenotypes during Mtz-based therapy, which is dictated by amounts of Mtz administered, frequency and duration of treatment, and gastric acidity or physiologic parameters that affect drug potency in *H. pylori*'s mucosal niche; and (iii) possible effects of resistance on *H. pylori* fitness during periods between therapy. Given the diversity among *H. pylori* strains and their human hosts, the evolutionary cost of a given level of Mtz resistance may depend on various aspects of bacterial genotype that affect the overall flow of metabolites during growth, and also on aspects of human genotype and physiology that affect human susceptibility to a given *H. pylori* strain (11, 12). Many of these issues should soon be clarified through high-resolution *H. pylori* molecular genetics and use of appropriate in vitro culture strategies and well-chosen experimental animal infection models.

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