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 to a prodrug to bactericidal agent. We found that (i) rdxA genes PCR amplified from 50 representative Mtz-resistant (Mtzs) strains from previously unstudied populations in Asia, South Africa, Europe, and the Americas could, in each case, transform Mtz-resistant H. pylori to Mtz-susceptible (Mtzs) strain resulting from mutation in rdxA; and (ii) transformation of Mtzs 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-susceptible, 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 water-borne pathogens (7, 8, 32, 38, 47). There is great intrinsic and public health interest in fully elucidating H. pylori’s metabolic pathways and how it 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.

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-resistant 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-resistant 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 H. pylori reference strain (26695); (ii) expression of rdxA from Mtz-resistant strains in Escherichia coli rendered this normally...

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Mtζ species Mtζ2; (ii) expression of a functional rdxA+ allele on a shuttle plasmid restored Mtζ susceptibility to an Mtζr H. pylori strain; and (iv) new mutations in rdxA, not gene transfer from unrelated lineages, were often responsible for Mtζ resistance in clinical isolates (16). In confirmation, rdxA mutations were found in 25 of 27 Mtζr derivatives of strain SS1 obtained from infected Mtζ-treated mice (22) and in 12 of 13 Mtζr clinical isolates from France and North Africa (42) (the bases of resistance in the unusual Mtζr strains with apparently intact rdxA genes were not determined). Consideration of enzyme mechanisms had indicated that Mtζ activation by the RdxA nitroreductase generates nitroso- and hydroxylamine-related compounds that should be mutagenic and bacterial (16).

Mtζ-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 Mtζ, an inadvertent consequence of therapy against other common infections, may induce as well as select for Mtζ resistance in this gastric pathogen.

Following our report linking rdxA inactivation and Mtζ resistance (16), several researchers suggested that other mechanisms (presumably rdxA independent) might often also cause Mtζ resistance (18, 27). This was based in part on observations that nominally Mtζr clinical isolates differ in the levels of Mtζ 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 Mtζ uptake or its active export (26, 40), (ii) more efficient DNA damage repair (6, 43), or (iii) enhanced scavenging of oxygen radicals that result from certain models of Mtζ activation (23, 41). Of particular note are plasmid- and transposon-borne nmr genes in certain Mtζr strains of Bacteroides fragilis that promote conversion of nitroimidazoles from produrg 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 European (Spanish) strains; 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 Mtζ 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 Mtζ 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 Mtζr (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 Mtζr phenotype of wild-type H. pylori strains. No evidence of determinants that bypass the need for rdxA inactivation in the development of significantly clinical Mtζ 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 are from the United Kingdom (44) from J99 from an ethnic European in Pulaski, Tenn. (T. L. Cover, personal communication).

H. pylori strains were grown on brain heart infusion agar (BHI) supplemented with 5% horse blood, 0.4% IsoVitaleX and the antibiotics amphotericin B (8 μg/ml), trimethoprim (5 μg/ml), and vancomycin (6 μg/ml). From this medium, cultures were 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% O2, 10% CO2, 85% N2). H. pylori transformation (5 μg/ml) was carried out as described elsewhere (34).

E. coli DH5α was grown on Luria-Bertani medium. The small multicopy Amp′ 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.

Determination of Mtζ sensitivity and resistance. Frozen H. pylori cultures were streaked onto Mtζ-free BHI agar and incubated for 3 days; then bacterial growth was respread on fresh Mtζ-free BHI agar and incubated for 1 day. The resulting colonies growing exponentially on plates were suspended in phosphates-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 Mtζ (variously, 0, 0.2, 0.5, 1.5, 3, 8, 16, and 32 μg/ml). When the frequency of cells that formed colonies on Mtζ-containing media was very low (<10−4), estimates of viability and mutant frequency were made more accurate by spotting 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 Mtζ 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 Mtζ for H. pylori (39), which would be exacerbated if H. pylori stressed by DNA-damaging agents tended to enter a hypermutable state (35).

New Mtζr mutants. Mtζr mutant derivatives of strain 26695 that may have increased resistance as well as selected by Mtζ (39) were obtained by plating linked cells from young cultures (as above) on BHI agar containing Mtζ at 3 μg/ml. Individual colonies were streaked on BHI agar with the same Mtζ concentration and then tested on BHI agar containing higher concentrations of Mtζ (6, 16, and 32, and 64 μg/ml).

Engineered H. pylori strains. (i) rdxA mutants. The rdxA::ampl allele, which contains a cat 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 (in a non-frameshift deletion in the 630-bp open reading frame) (rdxAΔ601) 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 EcoRV 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 rdxAΔ601 allele was introduced into Mtζ+ H. pylori strains by electroporation, with selection for Mtζ resistance on BHI agar with 3 or 8 μg of Mtζ/ml, with equivalent results. Mtζr 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 9-bp intron-frame deletion in rdxA (rdxAΔ111) was engineered, essentially as with rdxAΔ601, using outward-facing primers containing XbaI sites near their 5′ ends (rdxA-F3 and rdxA-R3). XbaI digestion of the linear PCR product, ligation,
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 ftxA::cam and ftxA::kan deletion mutants. The ftxA flavin nitroreductase gene segment (HP0642 in the strain 26695 genome [44]) was PCR amplified from H. pylori genomic DNA using primers ftxA-F1 and ftxA-R1 or, in some experiments, ftxA-F and ftxA-R. The amplified DNA were cloned into the EcoRV site of plasmid pBS and recovered after transformation of E. coli DH5α.

A marked 523-bp deletion in ftxA from strain 26695 was generated by PCR using outward-facing ftxA primers ftx-A-F2 and ftx-A-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 CamR (20 μg/ml) or KanR (20 μg/ml). The ftxA::cam and ftxA::kan alleles were introduced into H. pylori by transformation and selection for CamR (15 μg/ml) or KanR (20 μg/ml), as appropriate; the structures of transformants were verified by PCR with primers ftxA-F1 and ftxA-R1, as above.

(iii) HP1580X::insertion/deletion mutation. The HP1580 gene, which encodes a ferredoxin-like protein of unknown function, was PCR amplified from strain 26695 DNA, using primers 1508-F and 1508-R, and 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 CamR transformants were verified by PCR with primers 1508-F1 and 1508-R1, and similarly 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 structures of transformants were verified by PCR with primers 588-F1 and 588-R1, as above.

(iv) oorD::insertion/deletion mutation. The oorD (HP0588) gene, which encodes the ferredoxin 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 573-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 structures of transformants were 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 rdxA-F1 and rdxA-R1, and the cam cassette were cloned into the EcoRV and SmaI sites, respectively, of plasmid pBS (each gene transcribed toward the other). In parallel, a 1.37-kb segment of casA, PCR amplified from strain NCTC11637 using primers casA42143F and casA43512R, was cloned into the SmaI site of pBS. The resulting plasmid was linearized by PCR with outward-facing primers casA42673R and casA42900F, which are specific to sites 531 and 615 bp from the two ends of the cloned casA4 DNA fragment. The rdxA-cam segment was then 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 casA gene. An isolate in which rdx was oriented in the same direction as casA was used to transform NCTC11637 to CamR. The structures of resultant transformants were verified by PCR using primers casA42143F and casA43512R.

**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 ∼10⁻⁴ on BHI agar with 3 or 8 μg of Mtz/ml (phenotype designated 1R 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...
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' 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).

In equivalent characterizations of 55 representative Mtz' 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' mutants generated in culture.** To test our inference that Mtz resistance generally involves decreased rdxA function, new mutant Mtz' derivatives of reference strain 26695 were selected on BHI agar containing just 3 µg/ml, the lowest concentration of Mtz that allowed Mtz' mutants to emerge cleanly from background growth. Such mutants were obtained at frequencies of about 10⁻⁶ 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.

**Nature of newly arisen Mtz' mutants.** Eight low-level Mtz' 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' 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' 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' worldwide.** The idea that rdxA inactivation is critically involved in most 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' strains from various representative populations (12 Chinese, 12 Indian, 11 Alaska Native, 9 Peru Native, and 6 South African), electrophoresis of the Mtz' strain 26695 with these PCR-amplified rdxA DNAs, and quantitation of the yield of Mtz' transfectants on BHI agar with 8 µg of Mtz/ml (Fig. 2). Mtz' transfectants were
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 SS111, 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 ($\leq 10^{-4}$) (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 ($\approx 890$ 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 Camr 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 D601 or rdxA D111) (Fig. 3), in each case selected after DNA transformation and selection for resistance to just 3 mg 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 Mtzs 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.

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

<table>
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<tr>
<th>Mutant</th>
<th>Mtz phenotype</th>
<th>Nucleotide</th>
<th>Substitution</th>
<th>Amino acid*</th>
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<tr>
<td>202</td>
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<td>AAGGAA→AAAGAA</td>
<td>Shine-Dalgarno ribosome-binding site</td>
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* Position of the amino acid substitution is given in parentheses.

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**Hypothesis:** Mtz\textsuperscript{R} due to rdxA mutation

**Alternative:** Mtz\textsuperscript{R} due to other gene(s)

![Diagram showing DNA transformation strategy for testing involvement of rdxA gene mutation in Mtz\textsuperscript{R} clinical isolates.](http://jb.asm.org/)

**FIG. 2.** DNA transformation strategy for testing involvement of rdxA gene mutation in Mtz\textsuperscript{R} clinical isolates.
colony isolates (cultures) of each strain, with each culture assayed two times.

(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* (rdxa-deficient) *H. pylori* strain can be affected by other genetic determinants. First, introduction of the *rdxAΔ601* deletion into three derivatives of strain 26695 with weak Mtz* (3R 8S) 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 ~10^-2—that is, several hundred-fold higher than that of control *rdxAΔ601* transformants of 26695 wild type, selected in parallel (Fig. 3). This suggests that the mutations responsible for the 3R 8S phenotype may have affected processes 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 mutations 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 *rdxAΔ111* or *rdxAΔ601* deletion allele. In contrast, such hyper-resistant (64R) mutants were not obtained from 26695 wild type (rdxa*) (frequency, <10^-7). 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* clinical isolates that we screened had 32R or 64R phenotypes (Table 3). Transformants of strain 26695 made with *rdxa* genes from two hyper-resistant 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* phenotype (32R) (strain 439 in reference 16) that increased the yield of Mtz* *H. pylori* transformant colonies when mixed with *rdxA* mutant DNA but did not transform Mtz* *H. pylori* 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* *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* (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-
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' phenotype in each case, whereas equivalent transformation of the original rdxA' parental strains with the frxA::kan allele resulted in retention of Mtz' phenotypes. Thus, these two clinical isolates were unusual in requiring inactivation of both frxA and rdxA to achieve a clinically significant Mtz' 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 ~10^-5, in contrast to ~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' transformant derivatives of strain 26695 made in the rdxA' background had a normal 1.5R 3S phenotype; those in a rdxA4111 background had a normal 16R 32S phenotype; and those in an rdxA4111 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 ferrodoxin component of the multisubunit oxoglutarate oxidoreductase, an enzyme considered to be essential for viability (20). One Cam' 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' colony contained a replacement of the wild-type allele with the unknown function, was tested for possible effects on Mtz susceptibility or resistance after transformation with a HP1508::cam insertion allele. Cam' transformant derivatives of strain 26695 made in the rdxA' background had a normal 1.5R 3S phenotype; those in a rdxA4111 background had a normal 16R 32S phenotype; and those in an rdxA4111 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.

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**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 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-resistant 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-resistant strains. This is in accord with evidence that rdxA inactivation is sufficient to render Mtz-resistant strains Mtz-resistant. In this context, our finding that cloned frxA” genes from each of several Mtz-resistant 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-resistant 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 frxA (HP0642), an rdxA homolog that encodes a related reductase (24% amino acid sequence identity), 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.

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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 SS 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-resistant strains that were reported by others to contain normal rdxA sequences: 2 of 27 Mtz-resistant variants recovered from mice infected with strain SS1 and treated subtherapeutically with Mtz (22), and 1 of 13 Mtz-resistant strains from France and North Africa (42). It should now be possible to learn if any of these unusual mutants have decreased RxDA 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-resistant 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-resistant 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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