Roles of FrxA and RdxA Nitroreductases of *Helicobacter pylori* in Susceptibility and Resistance to Metronidazole

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The relative importance of the *frxA* and *rdxA* nitroreductase genes of *Helicobacter pylori* in metronidazole (MTZ) susceptibility and resistance has been controversial. Jeong et al. (J. Bacteriol. 182:5082–5090, 2000) had interpreted that Mtz^s *H. pylori* were of two types: type I, requiring only inactivation of *rdxA* to become resistant, and type II, requiring inactivation of both *rdxA* and *frxA* to become resistant; *frxA* inactivation by itself was not sufficient to confer resistance. In contrast, Kwon et al. (Antimicrob. Agents Chemother. 44:2133–2142, 2000) had interpreted that resistance resulted from inactivation either of *frxA* or *rdxA*. These two interpretations were tested here. Resistance was defined as efficient colony formation by single cells from diluted cultures rather than as growth responses of more dense inocula on MTZ-containing medium. Tests of three of Kwon's Mtz^s strains showed that each was type II, requiring inactivation of both *rdxA* and *frxA* to become resistant. In additional tests, derivatives of *frxA* mutant strains recovered from MTZ-containing medium were found to contain new mutations in *rdxA*, and *frxA* inactivation slowed MTZ-induced killing of Mtz^s strains. Northern blot analyses indicated that *frxA* mRNA, and perhaps also *rdxA* mRNA, were more abundant in type II than in type I strains. We conclude that development of MTZ resistance in *H. pylori* requires inactivation of *rdxA* alone or of both *rdxA* and *frxA*, depending on bacterial genotype, but rarely, if ever, inactivation of *frxA* alone, and that *H. pylori* strains differ in regulation of nitroreductase gene expression. We suggest that such regulatory differences may be significant functionally during human infection.

Helicobacter pylori is a genetically diverse bacterial species that chronically infects the stomachs of more than half of all people worldwide. Its long-term carriage is a major cause of chronic gastritis and peptic ulcer disease and is an early risk factor for gastric cancer (for reviews see references 5, 8, 28, and 32). Resistance to metronidazole (MTZ) is common and is important clinically as a primary cause of failure of MTZ-based anti-*Helicobacter* therapies (for reviews see references 10, 15, and 24). Frequencies of clinical isolates that are MTZ resistant range from only 10% in Japan (25) to 90% or more in India (26), and up to 50% or more of strains in the United States and Western Europe also are resistant (frequency varies among countries) (8, 23). These geographic differences probably reflect frequencies of MTZ use against other, mostly parasitic and anaerobic, infections and thus inadvertent MTZ exposure of resident *H. pylori* strains. Recent studies have implicated mutations in the chromosomal genes *rdxA* (HP0954) and *frxA* (HP0642) in the development of resistance (7, 9, 14, 16, 30, 35). These genes encode related nitroreductases that can convert MTZ from a harmless prodrug to products such as hydroxylamine that are both bactericidal and mutagenic (9, 29).

There has been disagreement about the quantitative contributions of *rdxA* and *frxA* to MTZ susceptibility and resistance. On the one hand, Kwon and associates had concluded that inactivation of either gene by itself could make any typical *H. pylori* strain resistant to MTZ (Mtz^r) (21), and that following

frxA inactivation, growth on MTZ-containing agar was not associated with mutation of *rdxA* (20). In contrast, we had concluded that *rdxA* inactivation is usually or always needed for a Mtz^s strain to become Mtz^r (16, 17). Two types of Mtz^s strains were distinguished, however, based on relative levels of FrxA nitroreductase activity. Most common were strains in which *rdxA* inactivation by itself was sufficient to cause resistance to moderate levels of MTZ (type I strains). Once *rdxA* had been inactivated in such type I strains, additional mutations that inactivated *frxA* (the second nitroreductase gene) resulted in higher levels of resistance, but *frxA* inactivation by itself had little, if any, effect on MTZ susceptibility if *rdxA* was still functional. Other strains, designated type II, required inactivation of both *frxA* and *rdxA* to become Mtz^r (16, 17). In contrast to Kwon et al. (21), we had not found any Mtz^s clinical isolate that was rendered Mtz^r by *frxA* inactivation alone.

Different protocols were used by Kwon et al. and ourselves to assess MTZ susceptibility or resistance. Their experiments relied on en masse growth responses after spotting aliquots of dense cultures (21), with resistance defined operationally as growth on the selective MTZ-containing medium (a traditional method for determining the minimal inhibitory concentrations [MIC] of antibacterial agents). Because up to about 0.01% of cells in fresh culture may form Mtz^r colonies on MTZ-containing medium (due to the mutagenic effects of MTZ, followed by selection for resistance [16, 29, and results of this work]), we considered a strain to be resistant only if the efficiencies of colony formation were unaffected by inclusion of MTZ in the medium. That is, cultures of interest were diluted, aliquots of each dilution were spotted, and the numbers of colonies

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TABLE 1. *H. pylori* strains

Strain ^a	Origin	Source and/or reference
Type I strains		
CAN9A	Peru	R. H. Gilman, 18
HK192	Hong Kong	B. Wong, 18
HUP57	Spain	M. Lopez Brea, 18
26695	United Kingdom	K. Eaton, 3, 31
Type II strains		
R10	South Africa	I. Segal, 18
SS1	Australia	A. Lee, 17, 22
X47 ^b	United States	H. Kleanthous, 19
2600	Texas	D. H. Kwon, 21
2667	Texas	D. H. Kwon, 21
2714	Texas	D. H. Kwon, 21
A219	Alaska (Cordova)	A. J. Parkinson
A381	Alaska (Nome)	A. J. Parkinson
A1099	Alaska (Kipnuk)	A. J. Parkinson
A10103	Alaska (Kotlik)	A. J. Parkinson

^a Type I and type II Mtz^s *H. pylori* strains are defined here as requiring inactivation of *rdxA* alone, versus requiring inactivation of both *rdxA* and *fixA*, to generate a Mtz^r phenotype (see the text and references 16 and 17).

^b Strain X47 is referred to as X47-2AL in reference 19.

formed at appropriate dilutions were scored on MTZ-containing and on MTZ-free media (12, 13).

The experiments presented here tested several possible explanations for the apparently different results and interpretations of Kwon et al. and ourselves, including (i) regulation of FrxA synthesis or activity by a quorum-sensing (cell density-dependent) mechanism, (ii) use of fundamentally different types of strains by our two groups, and (iii) use of dense rather than dilute cultures to score MTZ resistance, where interpretation of results with dense cultures might have been complicated by the mutagenicity of products of MTZ activation (21). Northern blot hybridization analyses were also carried out and showed that *fixA* mRNA and perhaps also *rdxA* mRNA were more abundant in type II than in type I strains.

MATERIALS AND METHODS

Bacterial strains and general methods. The Mtz^s *H. pylori* strains used here and their origins are listed in Table 1. *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) (referred to here as BHI agar), essentially as described previously (16, 17). MTZ was added when needed at concentrations appropriate to each experiment, as detailed below. Rifampin-resistant mutants were selected on medium with 5 µg of rifampin per ml. *H. pylori* cultures were incubated at 37°C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂).

Derivatives of these Mtz^s strains with null mutant alleles of the *rdxA* and *fixA* nitroreductase genes were constructed for the present experiments by electroporation, using standard *rdxA::cat* and *fixA::aph* or *fixA::cat* insertion mutant DNAs, with selection on BHI agar medium containing 15 µg of chloramphenicol per ml or 20 µg of kanamycin per ml, as described previously (16, 17). The genetic structures of transformants were checked by PCR to verify that they had resulted from allelic replacement using primers *rdxA*-F and *rdxA*-R or *fixA*-F1 and *fixA*-R1, as appropriate (see Table 2 for primer sequences). Mtz^r and Rif^r mutant frequencies were measured in at least four separate cultures, each derived from a different single transformant colony and each growing exponentially. Numbers or frequencies of resistant colonies reported are averages of four or more separate determinations. *H. pylori* genomic DNA isolation, PCR, and automated dye terminator cycle sequencing with *rdxA*-specific primers were carried out as described previously (16–18).

Determination of MTZ susceptibility and resistance. Frozen bacterial cultures were streaked on MTZ-free BHI agar and incubated for 3 days. Cells from one or a few colonies from these initial plates were then restreaked on fresh MTZ-

TABLE 2. Primers used

Primer	Sequence	Product size (bp) ^a
<i>rdxA</i> -F	5'-GCAGGAGCATCAGATAGTTCT	886
<i>rdxA</i> -R	5'-GGGATTTTATTGTATGCTACAA	
<i>rdxA</i> -F11	5'-CGCCATTCTTGCAAGATGTTTGA	542
<i>rdxA</i> -R11	5'-CTCGTTCTGCCACCCTCTT	
<i>rdxRT</i> -F	5'-GCATGCTGTGGTTGAATCTCAC	347
<i>rdxRT</i> -R	5'-CGAGCGCCATTCTTGCAAGATGT	
<i>rdxRT</i> -F	5'-GCATGCTGTGGTTGAATCTCAC	238
<i>rdxRT</i> -R2	5'-CCATGGCATTCTTGTATGGTTACT	
<i>fixA</i> -F	5'-GGATATGGCAGCCGTTTATCATT	780
<i>fixA</i> -R	5'-GAATAGGCATCATTTAAGAGATTA	
<i>fixA</i> -F11	5'-GCTTTACAGCACCAACGATTTG	617
<i>fixA</i> -R16	5'-TAAATAAATTCTGTCTTCCAGCGG	
<i>fixRT</i> -F	5'-GGACAGAGAACAAGTGGTTGCTT	341
<i>fixRT</i> -R	5'-GCGAACCTAGAATTAGTGTCAT	
<i>fixRT</i> -F2	5'-CTTCAATCGGGCTTGAACCATGGA	334
<i>fixRT</i> -R2	5'-GCTGCCATCATCATGTTCCGCAT	

^a Size of product from wild-type gene.

free BHI agar and incubated for one more day. The resulting exponentially growing cells were suspended in phosphate-buffered saline (PBS) buffer; a series of 10-fold dilutions of these cell suspensions was prepared, and 10 µl of each dilution was spotted on freshly prepared BHI agar containing various concentrations of MTZ (0, 0.2, 0.5, 1.5, 3, 5, 8, 16, 32, or 64 µg/ml). A strain was considered to be susceptible to concentrations of MTZ that caused at least a 10-fold decrease in the efficiency of colony formation by individual cells (efficiency of plating, or EOP). In our hands this use of aliquots from dilute bacterial cultures was more sensitive and reliable for scoring MTZ susceptibility and resistance of *H. pylori* than the traditional agar dilution method (16), which relies on growth versus nongrowth after spotting suspensions of at least >10⁵ cells on MTZ-containing medium. The results of a large clinical trial have led others to also conclude that neither agar dilution nor the popular Etest method is reliable for determining MTZ susceptibility or resistance in *H. pylori* (despite prior approval of agar dilution by the National Committee for Clinical Laboratory Standards) (27).

Much of the failure of these traditional methods can be attributed to the mutagenicity of products of MTZ activation: growth of *H. pylori* at concentrations that are close to the MIC for *H. pylori* can cause a more than 100-fold increase in new mutants among survivors (29 and Table 3). In practice, the spotting of 10⁵ Mtz^s cells on such MTZ-containing media often results in growth of new MTZ-induced Mtz^r mutants that could be misread as indicating resistance of all bacteria in the spot (16). Based on this understanding, we also did not attempt to assay MTZ susceptibility in liquid culture; any inference as to whether observed growth in liquid is due to all cells in the population or to just a subset of them is made tenuous by the combination of slow *H. pylori* growth and mutagenicity of products of MTZ activation.

When MTZ-resistant mutants were rare (<10⁻⁶) and accurate estimates of frequencies of resistant mutants were needed, culture aliquots were spread directly on the surface of an entire plate of MTZ-containing BHI agar rather than spotting smaller culture aliquots in a small area.

Throughout this report, the phenotype of a given strain or its mutant derivative is generally referred to with an R and S designation (e.g., 1.5R 3S) to refer to the maximum tolerated concentration of MTZ (in micrograms per milliliter) (R value) and the minimum MTZ concentration that will kill at least 99% of the culture (S value). The S value would generally correspond to MIC or minimal bactericidal concentration values that would be reported in traditional susceptibility testing of agents that are not mutagenic.

Viability and emergence of Mtz^r mutants in high-density cultures. Exponentially growing wild-type and *fixA* mutant *H. pylori* cells (1 × 10⁹ to 2 × 10⁹ cells) were suspended in PBS buffer and spread in 4- to 5-cm-diameter circular areas

TABLE 3. Frequency of rifampin-resistant mutants in *H. pylori* cultures^a

Strain	Pregrowth with MTZ (μg/ml) ^b	Survival ^c		No. of Rif ^r colonies/10 ⁸ cells	
		Wild type	<i>fxrA</i> null mutant	Wild type	<i>fxrA</i> null mutant
26695	0	1	1	10 ± 1	7 ± 2
	1	1	1	27 ± 10	6 ± 2
	2	10 ⁻²	10 ⁻²	93 ± 9	42 ± 10
	3	10 ⁻⁴	10 ⁻⁴	233 ± 37	61 ± 9
HUP57	0	1	1	6 ± 2	5 ± 1
	1	1	1	27 ± 6	5 ± 2
	2	10 ⁻¹	10 ⁻¹	64 ± 7	29 ± 6
	3	10 ⁻³	10 ⁻³	114 ± 43	38 ± 5
CAN9A	0	1	1	2 ± 1	2 ± 1
	1	1	1	7 ± 2	5 ± 2
	2	10 ⁻³	10 ⁻³	40 ± 19	25 ± 6
	3	10 ⁻⁴	10 ⁻⁴	49 ± 20	27 ± 6
HK192	0	1	1	4 ± 3	3 ± 1
	1	1	1	4 ± 2	3 ± 1
	2	10 ⁻²	10 ⁻²	19 ± 5	24 ± 3
	3	10 ⁻⁴	10 ⁻⁴	55 ± 9	28 ± 3
R10	0	1	1	2 ± 1	5 ± 2
	0.5	1	1	8 ± 3	16 ± 3
	1	10 ⁻²	10 ⁻²	106 ± 9	50 ± 9
	2	10 ⁻⁶	10 ⁻⁴	197 ± 32	58 ± 14

^a Data are the mean values and standard deviations of four independent cultures.

^b Pregrowth on medium with MTZ for 48 h before spreading on medium with rifampin.

^c Survival is defined as efficiency of colony formation on BHI agar with the indicated Mtz concentration.

on MTZ-containing (8 μg/ml) BHI medium. Each day cells were scraped from an untouched area within (not at the edges of) these large patches of bacterial growth and were suspended in PBS buffer at an optical density at 600 nm (OD₆₀₀) of 1.0. Ten-microliter volumes of suspension were then spotted directly or after serial dilution on MTZ-free or MTZ-containing BHI plates (0 and 8 μg of MTZ/ml) to measure overall bacterial titer (survival) and titer of resistant mutants. Mtz^r bacteria used for *rdxA* sequencing were obtained as pools or after purification of single colonies, as appropriate, on BHI agar with 8 μg of MTZ per ml.

mRNA analyses. To prepare RNA for reverse transcriptase (RT)-PCR (Fig. 1) or Northern blots (Fig. 2 and 3), cells growing exponentially on BHI agar were subcultured and grown for another 16 h, collected, and washed three times with 10% glycerol in distilled water at 0°C. Total RNA was isolated and prepared for RT-PCR and Northern hybridization essentially as described previously (2, 17) using a Qiagen RNeasy kit and treatment of RNA preparations with RNase-free DNase I (RT-PCR) or by phenol-chloroform and isoamyl extraction and ethanol precipitation (Northern hybridization). To check RNA quality, a few micrograms from each sample was electrophoresed in agarose, and the ethidium bromide-

FIG. 1. RT-PCR amplification of *rdxA* and *fxrA* gene segments from total RNA of type I and type II strains. The strains tested are as follows: lane 1, HK192; lane 2, 26695; lane 3, HUP57; lane 4, R10; lane 5, X47; lane 6, SS1. The primers *fxr*RT-F2 and *fxr*ART-R2 were used to amplify *fxrA* gene segments (334 bp). The primers *rdx*RT-F and *rdx*RT-R2 were used to amplify *rdxA* gene segments (238 bp).

FIG. 2. DNA sequence of probe affects efficiency of detection of transcripts. RNAs from seven strains were electrophoresed and blotted for Northern analysis in triplicate and probed with *rdxA* probe DNAs prepared from each of three different strains. The strains used for RNA (and in three cases, DNA probe) preparation were as follows: lane 1, 26695; lane 2, HK192; lane 3, HUP57; lane 4, SS1; lane 5, R10; lane 6, X47; lane 7, 2667 (strains in lanes 1 to 3 are type I and strains in lanes 4 to 7 are type II). 23S and 16S rRNAs were visualized after being stained with methylene blue (23S rRNA shown in bottom panel) to ensure that the RNA was of good quality and that equal amounts were loaded in each lane.

stained gel was inspected to make sure that the ratio of intensities of 23S and 16S rRNAs was about 2:1 and that there was little, if any, accumulation of the small RNA fragments (probably degradation products) that run just ahead of the 0.2-kb RNA size marker (Perfect RNA Markers, 0.2 to 10 kb; Novagen, Madison, Wis.).

For RT-PCR experiments, an absence of genomic DNA contamination was verified by PCR using *Taq* DNA polymerase without reverse transcriptase. Then RT-PCR was carried out with 10 ng of RNA using the One-Step RT-PCR kit (Gibco BRL) with primers *fxr*RT-F and *fxr*RT-R (for *fxrA* mRNA) and *rdx*RT-F and *rdx*RT-R (for *rdxA* mRNA).

For Northern blot analyses 10 μg of RNA was separated by electrophoresis on a 1.5% agarose-6% formaldehyde morpholinepropanesulfonic (MOPS) gel, along with RNA size markers, as above. RNA in the gel was blotted to a Hybond N⁺ nylon membrane (Amersham) by capillary transfer. DNA fragments used both as probes and as hybridization standards (described below) were generated by PCR from genomic DNA using primer pairs *fxrA*-F11 and *fxrA*-R16 for *fxrA* probes (617 bp) and *rdxA*-F11 and *rdxA*-R11 for *rdxA* probes (541 bp) (Table 1). With each probe, 1 μg of PCR product was labeled with digoxigenin-11 dUTP (DIG) using the DIG High Prime kit (Roche Diagnostics Corporation, Indianapolis, Ind.), and the concentration of labeled product was estimated as recommended by the manufacturer. To avoid effects of base sequence divergence on efficiency of RNA-DNA hybridization (see Fig. 2 and Results), each probe used in the experiment summarized in Fig. 3 was constructed to be strain specific. That is, the probe was generated by PCR from genomic DNA of the strain whose RNA transcripts were to be studied. Eighteen separate probes were prepared in this way, one for *rdxA* and the other for *fxrA* from each of nine strains.

To prepare hybridization standards, aliquots of *rdxA* and *fxrA* PCR products from each strain were adjusted to 1 ng/μl, and then aliquots containing 1, 10, and 100 pg of PCR product were spotted as hybridization standards on nylon membranes. Each series of standards was then included in the hybridization sack with its cognate Northern RNA blot and DIG-labeled hybridization probe (concentration of 100 to 200 ng of probe per μl). Hybridization was carried out at 50°C using DIG Easy Hyb buffer (Roche Diagnostics), the filters were washed twice for 15 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate at 53°C, and hybridization was detected using a DIG Luminescent Detection kit with CDP-star chemiluminescent substrate (Roche Diagnostics). The hybridized Northern blot and DNA standards were arrayed and exposed for times ranging from 1 to 5 s. Figure 3 was prepared as a composite from various hybridization exposure times, chosen based on giving approximately equal intensities in hybridization standards, thereby allowing direct comparison of *fxrA* and *rdxA* mRNA levels among the strains tested.

RESULTS

We tested several possible explanations for the apparently different results and proposed mechanisms of MTZ resistance: *fxrA* inactivation is a major cause of MTZ resistance (21)

FIG. 3. *frxA* and *rdxA* mRNA transcript levels in type I and type II strains detected with strain-specific probes. Each of the 18 RNA transcripts (*rdxA* and *frxA* in each of nine strains) was detected with a probe generated by PCR from genomic DNA from the corresponding strain and DIG labeling. Small differences in amounts or activity of labeled probe were compensated for by using the probe DNA itself as an internal standard and varying exposure for times, ranging from 1 to 5 s, in order to allow direct comparison of amounts of *frxA* and of *rdxA* mRNA among the strains tested. The three type I strains tested in this way were as follows: lane 1, 26695; lane 2, HK192; lane 3, HUP57. The six type II strains tested were as follows: lane 4, SS1; lane 5, R10; lane 6, X47; lane 7, 2600; lane 8, 2667; lane 9, 2714.

versus *frxA* inactivation by itself rarely, if ever, confers resistance (16).

Effect of *frxA* on mutation frequency. One possible explanation of previous results (20, 21) holds that *frxA* inactivation rendered *H. pylori* highly mutable when grown at high cell

density, conceivably by perturbing metabolite balances that, in turn, decreased the fidelity of DNA replication or potency of DNA repair (see, e.g., reference 11). This was tested by measuring frequencies of rifampin-resistant mutants (which generally result from point mutations in an RNA polymerase gene [13]) in cultures of five wild-type MTZ-susceptible strains and in their isogenic *frxA* knockout mutant derivatives. Table 3 shows that *frxA* inactivation had no significant effect on the frequency of spontaneous mutants, and furthermore that it decreased the yield of MTZ-induced mutants several-fold. Thus, the postulated development of a *Mtz^r* phenotype by *frxA* inactivation (21), if correct, would not be due to *H. pylori* entry into a hypermutable state.

Kinetics of MTZ killing at high density. Several MTZ-susceptible wild-type strains and their *frxA* knockout mutant derivatives were used to test a model in which incubation at high cell density would render *H. pylori* tolerant or resistant to MTZ phenotypically, without additional mutation. This entailed inoculation of MTZ-susceptible bacteria at high cell density on MTZ-containing medium, scraping them from the agar surface after one or more days of incubation, and determining bacterial titers on media with and without MTZ. The results (Table 4) showed that all cells that would be considered MTZ-susceptible genetically (unable to form isolated colonies on MTZ-containing medium) were also killed eventually during incubation at high density with MTZ. The kinetics of cell killing depended on both background genotype and whether *frxA* was functional or not. For example, strain 26695 (type I) was killed more rapidly than HUP57 (also type I) and X47 (type II) (compare titers in Table 4 for day 1, 0 μ g of MTZ per ml).

The lack of persistent physiologic resistance without further mutation in bacteria incubated at high density was illustrated by finding that the only cells to survive well on MTZ-containing medium also formed individual *Mtz^r* colonies when replated after appropriate dilution (the identification of new *rdxA* mutations in such derivatives is described below). Such resistant mutants became abundant after only 1 day in the case of strain 26695 and after 2 days in the case of HUP57 (Table 4). Resistant derivatives of strains SS1 and X47 were not found; this probably reflects a need for mutation in two genes if these strains are to become resistant (16, 17, and below). Inactivation of *frxA* slowed MTZ-induced killing in all cases, thereby weak-

TABLE 4. *H. pylori* survival and accumulation of *Mtz^r* mutants on MTZ-containing medium

Mtz ^s strain	Titers of <i>H. pylori</i> recovered from MTZ-containing medium on the indicated day and concn (in μ g/ml) of MTZ (day [concn]) ^a					
	day 1, MTZ (0)	day 1, MTZ (8)	day 2, MTZ (0)	day 2, MTZ (8)	day 3, MTZ (0)	day 3, MTZ (8)
Type I						
26695 WT	1.5×10^4	1.0×10^4	3.1×10^4	3.2×10^4	3.1×10^5	2.8×10^5
26695 <i>frxA</i>	2.4×10^4	3.4×10^4	3.6×10^4	4.5×10^4	5.3×10^5	4.2×10^5
HUP57 WT	5.9×10^5	7.0×10^2	1.0×10^3	5.0×10^2	2.3×10^2	3.2×10^2
HUP57 <i>frxA</i>	4.2×10^6	2.0×10^3	1.2×10^6	1.9×10^3	2.2×10^4	1.6×10^4
Type II						
SS1 WT	1.5×10^3	None	None	None	None	None
SS1 <i>frxA</i>	5.6×10^6	2.0×10^2	3.3×10^6	3.0×10^2	4.0×10^3	3.3×10^3
X47 WT	1.7×10^5	None	5.0×10^2	None	None	None
X47 <i>frxA</i>	4.0×10^5	4.8×10^2	5.0×10^4	1.9×10^2	3.9×10^2	1.6×10^2

^a Bacterial suspensions were adjusted to an OD₆₀₀ of 1.0 and the numbers of CFU per 10- μ l aliquot were determined. The titers of *H. pylori* from drug-free plates (controls) were typically 1×10^7 to 2×10^7 cells per 10 μ l per OD₆₀₀. WT, wild type.

TABLE 5. *rdxA* alleles from *frxA* mutant *H. pylori* recovered from MTZ-containing BHI agar^a

Strain	Mtz ^r isolate	Change in:	
		DNA	Protein
Type I			
26695 <i>frxA::cat</i>	1	AG insertion	Frameshift from Glu(138)
	2	GAG→TAG	Glu→stop(75)
	3	CGC→CAC	Arg→His(16)
HUP57 <i>frxA::aph</i>	1	C insertion	Frameshift from Cys(159)
	2	CAG→TAG	Gln→stop(50)
	3	GA deletion	Frameshift from Glu(107)
CAN9A <i>frxA::aph</i>	1	CAA→TAA	Gln→stop(102)
	2	GAAATCGCT insertion	Insertion of 3 amino acids (Glu, Met, and Ala) from 41
	3	TGG→TGA	Trp→stop(209)
HK192 <i>frxA::aph</i>	1	T insertion	Frameshift from Leu(157)
	2	T insertion	Frameshift from Met(84)
	3	A deletion	Frameshift from Lys(64)
Type II			
R10 <i>frxA::aph</i>	1	GAA→TAA	Glu→stop(32)
	2	GAA→TAA	Glu→stop(32)
	3	GAG→TAG	Glu→stop(75)

^a *H. pylori* were recovered from BHI agar containing 8 µg of MTZ per ml. The genes *aph* and *cat* confer resistance to kanamycin and chloramphenicol, respectively.

ening the selection for MTZ resistance. Nevertheless, with *frxA* mutants, 2 (26695 lineage) or 3 days of incubation on MTZ-containing medium was sufficient to kill most or all cells that had not also become MTZ resistant. Thus, these outcomes argue against the idea that resistance is caused by incubation at high cell density.

Mutation in *rdxA* needed for *frxA* mutant strains to become Mtz^r. We next tested the suggestion (20) that Mtz^r derivatives of *frxA* knockout mutant strains selected initially at high cell density rarely, if ever, carried new mutations in *rdxA*. Mtz^r mutants were selected by spreading cells from dense cultures of *frxA* knockout mutant derivatives of four type I lineages and one type II lineage on MTZ-containing agar, as above, and incubating them for three days. Mtz^r colonies formed by single cells were obtained later by streaking diluted aliquots of the dense suspensions of Mtz^r cells on new MTZ-containing agar plates. *rdxA* was PCR amplified from cell suspensions incubated on MTZ-containing agar and from single Mtz^r colonies, and the products were sequenced. Table 5 shows that each Mtz^r single colony isolate contained a sequence change in *rdxA*, resulting variously in frameshifts, translation stop codons, or amino acid additions or replacements that would also likely inactivate the gene. In contrast, the *rdxA* sequence profiles obtained after selecting confluent lawns of Mtz^r mutant bacteria matched those from the wild-type parent (data not shown). This might be due to the presence of numerous independent Mtz^r mutants in each bacterial pool. In this case, the sequence change in any single mutant allele would be obscured by the many other sequences containing unchanged nucleotides at that site (only when cultures contain just one or a few abundant mutants can each allele be detected reliably). Alternatively, it might reflect DNA extraction before new Mtz^r mutants had adequately outgrown their Mtz^s siblings (e.g., as shown in Table 4). Either interpretation might explain the seeming wild-type *rdxA* sequence in *H. pylori* collected from MTZ-containing agar that was reported previously (20).

Type II Mtz^s strains and *frxA*. Three of the clinical isolates whose initial studies had led Kwon and colleagues (20) to conclude that *frxA* inactivation by itself caused MTZ resistance, strains 2600, 2667, and 2714 (kindly provided by D. H. Kwon and D. Y. Graham) were studied and found to each be highly susceptible to MTZ. In particular, as with type II reference strains from elsewhere, Mtz^r mutant colonies were found at frequencies of $\leq 10^{-8}$ on medium with 8 µg of Mtz per ml (see Table 6). In contrast, Mtz^r mutant colonies were found in cultures of type I strains at frequencies of about 10^{-4} (16, 17). There was some variability among type II strains in survival on

TABLE 6. Survival of Mtz^s *H. pylori* clinical isolates on MTZ-containing agar^a

Strain	Survival on agar containing the following concn of MTZ (µg/ml)				
	1	1.5	3	5	8
Experimental (type II)					
2667	1	10 ⁻³	10 ⁻⁶	10 ⁻⁷	<10 ⁻⁸
2714	1	10 ⁻³	10 ⁻⁶	10 ⁻⁷	<10 ⁻⁸
2600	1	10 ⁻⁴	10 ⁻⁶	10 ⁻⁷	<10 ⁻⁸
X47	1	10 ⁻³	10 ⁻⁶	ND ^b	<10 ⁻⁸
A1099	1	10 ⁻⁴	10 ⁻⁶	10 ⁻⁷	<10 ⁻⁸
A381	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	<10 ⁻⁸
A10103	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	ND	<10 ⁻⁸
SS1	10 ⁻⁴	10 ⁻⁶	<10 ⁻⁸	<10 ⁻⁸	<10 ⁻⁸
R10	10 ⁻⁴	10 ⁻⁶	10 ⁻⁶	ND	<10 ⁻⁸
A219	10 ⁻⁵	10 ⁻⁶	<10 ⁻⁸	ND	<10 ⁻⁸
Control (type I)					
26695	1	1	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴
CAN9A	1	1	10 ⁻⁴	ND	10 ⁻⁴
HK197	1	1	10 ⁻⁴	ND	10 ⁻⁵
HUP57	1	1	10 ⁻⁴	ND	10 ⁻⁴

^a EOP, were determined as described in Materials and Methods, at least four times for each strain, each time with a separate culture.

^b ND, not determined.

medium with lower levels of MTZ (e.g., with 3 µg of MTZ per ml, $\sim 10^{-6}$ with each of D. H. Kwon's strains and five of the seven other type II strains, but $\sim 10^{-8}$ with the other two; Table 6). This variability is in accord with the considerable genetic diversity among *H. pylori* strains, as will be detailed below.

To further examine the roles of nitroreductase genes in the three strains from Kwon and colleagues, new derivatives of these strains with *rdxA* and *frxA* null mutations were generated and tested for susceptibility or resistance to MTZ (as detailed in Materials and Methods) (Table 7). We found that each strain remained susceptible to 8 µg of MTZ per ml after inactivation of either one of these genes, as did other type II clinical isolates. In contrast, inactivation of both genes conferred high-level resistance (32 µg per ml) in each case. It is also important that inactivation of either nitroreductase gene dramatically increased the EOP on MTZ-containing agar, i.e., the ease of recovery of new *Mtz^r* mutants (from about $\leq 10^{-8}$ to about 10^{-4}) (Table 7). This is in accord with the need for one new gene mutation in derivatives of type II strains that already lack *rdxA* or *frxA* function versus the need for two independent gene mutations in their wild-type (type II) parents.

We also note that *rdxA* inactivation caused small increases in the intrinsic low-level resistance to MTZ in 4 of the 10 type II strains studied (resistant to 3 or 5 but not 8 µg of MTZ per ml; see Table 7), whereas *frxA* inactivation had little, if any, effect on this low-level MTZ resistance. Since inactivation of both *rdxA* and *frxA* increased resistance to the same level in each of these strains (to 32 µg of MTZ per ml; see Table 7), we infer that FrxA activity varies among type II strains. For example, it seemed lower in strains such as 2714 and A1099 than in A219 and SS1, since *rdxA* mutant derivatives of 2714 and A1099 seemed resistant to slightly higher levels of MTZ (5R versus $\leq 1R$ in phenotype).

***frxA* mRNA is more abundant in type II than in type I cells.** Initially we sought to use RT-PCR (as in reference 17) to test the hypothesis that *frxA* mRNA is more abundant in type II than in type I strains. Although results with one type II and one type I strain (SS1 and 26695) had supported this view (17 and Fig. 1), results with other strains were erratic. In particular, no *frxA* RT-PCR product was detected from strains R10 and X47, using *rdxA* mRNA as an internal standard (RT-PCR with *frxA* and *rdxA* primers together), despite mutational evidence showing that acquisition of *Mtz^r* in these strains depended on inactivation of *frxA* as well as *rdxA* (Table 7). Various ratios of *frxA* and *rdxA* RT-PCR products were obtained from several other type II strains, and one type II strain did not seem to yield an *rdxA* RT-PCR product at all (see Fig. 1). These unexpected results were obtained repeatedly in trials with RNA preparations from different exponentially growing cultures of the same strain. Accordingly, much of the variation among strains in RT-PCR results was ascribed provisionally to effects of sequence (e.g., secondary structure in single-stranded nucleic acids) on efficiency of amplification rather than differences in levels of target mRNAs.

Northern blot hybridization tests were initiated next to avoid complications that might stem from sequence-dependent differences in amplification efficiency. Preliminary experiments indicated, however, that any given probe tended to detect *rdxA*

TABLE 7. Effects of *rdxA* and *frxA* inactivation on *H. pylori* phenotype^a

Strain	R and S values (in µg/ml) for:			
	Wild type	<i>rdxA::cat</i>	<i>frxA::aph</i>	<i>rdxA::cat</i> <i>frxA::aph</i>
Type II strain ^b				
A219	0.5R 1S	1R 1.5S	0.5R 1S	32R 64S
SS1	0.5R 1S	1R 1.5S	1R 1.5S	32R 64S
R10	0.5R 1S	1R 1.5S	1R 1.5S	32R 64S
A10103	0.5R 1S	1.5R 3S	0.5R 1S	32R 64S
X47	1R 1.5S	1.5R 3S	1.5R 3S	32R 64S
2600 (D. K.) ^c	1R 1.5S	1.5R 3S	1.5R 3S	32R 64S
A381	0.5R 1S	3R 5S	0.5R 1S	32R 64S
2667 (D. K.)	1R 1.5S	3R 5S	1.5R 3S	32R 64S
2714 (D. K.)	1R 1.5S	5R 8S	1R 1.5S	32R 64S
A1099	1R 1.5S	5R 8S	1R 1.5S	32R 64S
Type I control strain				
26695	1.5R 3S	16R 32S	1.5R 3S	32R 64S

^a R (as in 1R, 1.5R, etc.) refers to maximum concentration of MTZ that the *H. pylori* strain can tolerate; S (as in 1.5S, 3S, etc.) refers to minimum concentration of MTZ that will kill at least 99% of the culture and would correspond to the MIC value that is reported in traditional susceptibility testing of antimicrobial agents that are not mutagenic.

^b The type II strains are grouped according to the change in resistance level that resulted from *rdxA* inactivation. This, we infer, is related inversely to the level of *frxA* expression.

^c Strains studied by D. H. Kwon and associates (21) are identified with (D. K.).

mRNA from the same strain more efficiently than from other *H. pylori* strains. For example, the quite abundant *rdxA* RNA transcript of the strain labeled 2 in Fig. 2 was detected efficiently only with a DNA probe generated from that strain, not with probes generated from the strains labeled 4 or 6. Similarly, *rdxA* mRNA from strain 6 was detected more efficiently with the probe from strain 6 than that from 2, and the probe from strain 4 was useful only for detecting the *rdxA* transcript from 4. These outcomes implied that efficiencies of DNA-RNA hybridization were also affected by sequence divergence among *H. pylori* strains and that interstrain comparisons based on just one gene-specific probe would be unreliable.

To bypass effects of sequence divergence on efficiencies of hybridization, *frxA* and *rdxA* probes specific to (generated from the genomic DNA of) each strain of interest were prepared. To allow direct comparisons of hybridization data from each of nine strains tested (18 separate hybridizations, each with a separately prepared probe), fixed amounts of each *rdxA* or *frxA* probe DNA were spotted on separate filters and included as internal standards in each hybridization (for details see Materials and Methods and the legend to Fig. 3). We found that *frxA*-containing transcripts (Fig. 3A, arrow) were abundant in each of the six type II strains studied, including several that had given erratic results in RT-PCR, whereas *frxA* transcripts were less abundant or were absent from each of the three type I strains tested. *rdxA* transcripts were found in all strains tested, as expected, although they seemed to be slightly (perhaps two-fold) more abundant in type II than in type I strains.

It is also noteworthy that the transcripts were each longer than could be expected of monocistronic transcription units (about 2.2 kb versus 651 bp for the *frxA* open reading frame and about 1.4 kb versus 630 bp for the *rdxA* open reading frame), implying that each is polycistronic. Comparison of the

two fully sequenced *H. pylori* genomes revealed a polymorphism for the presence or absence of a ~1-kb segment just upstream of *frxA* (present in strain J99; absent from strain 26695 [4, 31]). PCR tests indicated that this 1-kb segment is present in two of the three type I strains and in each type II strain studied here (data not shown). Thus, our results do not suggest that the presence or absence of this upstream region regulates *frxA* expression and MTZ susceptibility.

DISCUSSION

The present experiments, initiated to test two alternative views of how *H. pylori* becomes resistant to MTZ, have given new insights into the genetic diversity of this species. Two types of MTZ-susceptible strains were identified by genetic (mutational) and molecular (Northern blot) tests. Those designated type I needed inactivation only of *rdxA* to become resistant, apparently because only the *rdxA* nitroreductase gene was well expressed. Type II strains, in contrast, needed inactivation of both *rdxA* and the related *frxA* gene to become resistant, apparently because each gene was highly active. These two Mtz^s strain types were also distinguished by frequencies of MTZ-resistant mutants: about 10⁻⁴ and 10⁻⁸ in cultures of type I and type II strains, respectively, reflecting the need to inactivate just one gene (*rdxA*) versus two genes (*rdxA* and *frxA*) to become resistant. Our tests also showed that *frxA* inactivation slowed the killing by MTZ of type I strains with functional *rdxA* genes and increased the resistance of type I derivatives that were already mutated in *rdxA* (although *frxA* inactivation by itself did not confer MTZ resistance if *rdxA* remained functional). These subtle effects of *frxA* inactivation implied that *frxA* can be expressed in type I strains, although too weakly for detection in our Northern blots. Given the initial aim of our study, it is noteworthy that these results were also obtained with strains used by another group (21) that had interpreted that *frxA* inactivation by itself could render *H. pylori* MTZ resistant. The present results suggest that their interpretation is incorrect.

We ascribe the differences in interpretation to technical aspects of how susceptibility versus resistance was scored. We monitored the susceptibility or resistance of individual cells to MTZ by diluting cultures sufficiently to count colonies formed on media with and without MTZ. This quantitative plating method, although common in bacterial genetics, contrasts with traditional practice in clinical microbiology of scoring (i) growth responses of spots of dense cell suspensions on medium containing the antibiotic (as in reference 21) or (ii) zones of growth inhibition near antibiotic-containing disks or Etest strips laid on dense bacterial lawns. The special value of quantitative plating tests when scoring MTZ resistance stems from the mutagenicity of MTZ and consequently the high frequency of MTZ-resistant mutants (10⁻⁴) when mutation in only one gene is needed. This high frequency is enough to sometimes give a misleading appearance of resistance in traditional qualitative en masse growth tests (as in reference 21), a problem that may be exacerbated by the delayed killing of strains that are MTZ susceptible but contain *frxA* null alleles. Such traditional MTZ resistance tests were also deemed unreliable in a recent large clinical trial (27).

A second technical feature of our experiments also merits

comment: the need for strain-specific hybridization probes when comparing transcript levels in different strains. Although initial RT-PCR with strains 26695 (type I) and SS1 (type II) had demonstrated *frxA* mRNA only in SS1 (17) as expected, RT-PCR with other type II strains often revealed little, if any, *frxA* product. Erratic results were also obtained when Northern hybridization with a single probe was used to monitor levels of *rdxA* mRNA in several strains (Fig. 2). These problems can be ascribed to sequence diversity among *H. pylori* strains (typically some 4 to 6% in loci such as *rdxA* and *frxA*) and consequent differences in relative efficiencies of amplification and hybridization between imperfectly matched probe and transcript in Northern blots. Accurate Northern blot results were obtained by generating strain-specific gene probes for each strain of interest, labeling each probe individually, and using each in a separate hybridization. Internal DNA calibration standards were included in each hybridization to allow valid quantitative interstrain comparisons.

Much remains to be learned about the metabolic phenomena that affect MTZ susceptibility and resistance, including the mechanisms underlying the diversity among strains in apparent levels of the related nitroreductases studied here and the actual roles of each nitroreductase during human gastric infection. In particular, although strains with highly expressed *frxA* genes as defined here seem to be uncommon ($\leq 10\%$ of Mtz^s isolates) in many populations (16), we have recently found that about half of Mtz^s strains from Lithuania are of this type (G. Dailide, D. Kersulyte, D. Dailidienė, J. Miciuleviciene, and D. E. Berg, unpublished data). Neither the factors underlying this geographic difference nor its generality (i.e., in other Baltic or Northern European countries) are known. That the type I versus type II difference might be adaptive and significant physiologically, however, is suggested by a recent finding that *frxA* mutants of the type II strain SS1 grow less well in mice than does the wild type (J. Y. Jeong, D. Dailidienė, A. K. Mukhopadhyay, and D. E. Berg, unpublished data). We are attracted to the possibility that the observed differences in gene or enzyme activity may often be significant physiologically for *H. pylori*—perhaps affecting bacterial nutrition and the persistence of infection in various human hosts.

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