Metronidazole Activation Is Mutagenic and Causes DNA Fragmentation in *Helicobacter pylori* and in *Escherichia coli* Containing a Cloned *H. pylori rdxA*⁺ (Nitroreductase) Gene

GARY SISSON,[1] JIN-YONG JEONG,[2] AVERY GOODWIN,[1] LOUIS BRYDEN,[1] NORMA ROSSLER,[1]
SABRINA LIM-MORRISON,[1] AUSRA RAUDONIKIENE,[1] DOUGLAS E. BERG,[2]
AND PAUL S. HOFFMAN[1,3]*

Department of Microbiology and Immunology[1] *and Division of Infectious Diseases, Department of Medicine,*[3]
*Dalhousie University, Halifax, Nova Scotia, Canada, and Department of Molecular Microbiology and
Department of Genetics, Washington University School of Medicine, St. Louis, Missouri*[2]

Much of the normal high sensitivity of wild-type *Helicobacter pylori* to metronidazole (Mtz) depends on *rdxA* (HP0954), a gene encoding a novel nitroreductase that catalyzes the conversion of Mtz from a harmless prodrug to a bactericidal agent. Here we report that levels of Mtz that partially inhibit growth stimulate forward mutation to rifampin resistance in *rdxA*⁺ (Mtz^s) and also in *rdxA* (Mtz^r) *H. pylori* strains, and that expression of *rdxA* in *Escherichia coli* results in equivalent Mtz-induced mutation. A reversion test using defined *lac* tester strains of *E. coli* carrying *rdxA*⁺ indicated that CG-to-GC transversions and AT-to-GC transitions are induced more frequently than other base substitutions. Alkaline gel electrophoretic tests showed that Mtz concentrations near or higher than the MIC for growth also caused DNA breakage in *H. pylori* and in *E. coli* carrying *rdxA*⁺, suggesting that this damage may account for most of the bactericidal action of Mtz. Coculture of Mtz^s *H. pylori* with *E. coli* (highly resistant to Mtz) in the presence of Mtz did not stimulate forward mutation in *E. coli*, indicating that the mutagenic and bactericidal products of Mtz metabolism do not diffuse significantly to neighboring (bystander) cells. Our results suggest that the widespread use of Mtz against other pathogens in people chronically infected with *H. pylori* may stimulate mutation and recombination in *H. pylori*, thereby speeding host-specific adaptation, the evolution of virulence, and the emergence of resistance against Mtz and other clinically useful antimicrobials.

Metronidazole (Mtz) [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] and related 5-nitroimidazoles are redox-active prodrugs that are often used to treat infections caused by anaerobic bacteria and protozoa (7, 18, 28). They are also a key component of combination therapies that are used to eradicate *Helicobacter pylori*, the microaerophilic bacterium which chronically infects the stomachs of more than half of all people worldwide and is the major cause of peptic ulcer disease and an early risk factor for gastric cancer (3, 14, 20, 30). In anaerobes, redox-active enzymes such as pyruvate/ketoacid oxidoreductases and hydrogenase, active with the low-redox carriers (ferredoxin and flavodoxin), reduce 5-nitroimidazoles to mutagenic products that also cause DNA helix destabilization and single- and double-strand DNA breakage (7, 18, 19, 28). High-level Mtz resistance is rare in anaerobes, because the activating enzymes are essential components of core metabolic pathways and because these microbes generally contain cytoplasmic components of very low redox potential that can spontaneously activate the drug (7, 19, 23, 28). In contrast, moderate to high-level resistance to nitroimidazoles is common among *H. pylori* clinical isolates, with frequencies ranging from ~10 to 90% or more of strains, depending on geographic region (12). These frequencies generally reflect the incidence of Mtz usage against other (parasitic or anaerobic bacterial) infections in particular societies (6, 10).

We have found that (i) nearly all Mtz-resistant (Mtz^r) *H. pylori* clinical isolates contain loss-of-function mutations in *rdxA* (HP0954 in reference 27), an *H. pylori* gene that encodes a nonessential oxygen-insensitive NADPH nitroreductase (RdxA); (ii) mutational inactivation of *rdxA* is both necessary and sufficient to confer an Mtz^r phenotype; and (iii) expression of the *H. pylori rdxA*⁺ gene in *E. coli* renders this normally Mtz^r bacterium susceptible to Mtz (5, 9, 17). Several other studies have confirmed an association between loss-of-function mutations in *rdxA* and Mtz resistance (15, 26). RdxA, a homolog of the classical nitroreductases of enteric bacteria, exhibits NADPH-dependent Mtz reductase activity and a substrate preference for Mtz that may be related to its unique cysteine-rich nature and alkaline pI (9). The stoichiometry of the RdxA-catalyzed reduction of Mtz indicates an anaerobic two-step (four-electron) reduction of the 5-nitro group of Mtz to mutagenic and DNA-damaging nitroso and hydroxylamine products. Although the DNA sequences of *rdxA* genes from unrelated strains typically differed at some 5% of nucleotide positions (as is quite typical of housekeeping genes in *H. pylori*), the *rdxA* genes from related Mtz^r and Mtz^s strains recovered from infections that were mixed (Mtz^r, Mtz^s), but uniform in overall DNA fingerprint type, differed by only one or a few base pairs. This indicated that Mtz resistance tends to result from de novo mutation in the resident *rdxA* gene, rather than from lateral transfer of mutant *rdxA* (or other) genes from unrelated but Mtz^r strains. Equivalent results were obtained with a dozen patients from France and North Africa (26). These findings suggested that Mtz-based therapies might be mutagenic, inducing as well as selecting for Mtz resistance in *H. pylori* (9).

* Corresponding author. Present address: Anti Infectives Research Division, SmithKline Beecham Pharmaceuticals, 1250 Collegeville Road, Collegeville, PA 19426. Phone: (610) 917-6010. Fax: (610) 917-7901. E-mail: Paul_2_Hoffman@sbphrd.com or phoffman@tupdean2.med.dal.ca.

Here we show directly that products of Mtz activation are mutagenic and DNA damaging in *H. pylori* and in *Escherichia coli* containing a cloned *rdxA*$^+$ gene. These findings have implications for the emergence of resistance to various antibacterial agents and more generally to the evolution of virulence and the adaptation of *H. pylori* to individual hosts.

MATERIALS AND METHODS

Bacterial strains and DNA manipulations. *H. pylori* Mtz^s strains 950 and 1134 and Mtz^r strain 1134R have been described elsewhere (9). Strain 26695 (27) and its Mtz^r (*rdxA* deletion mutant) derivatives 16R and 64R (carrying null mutations in *rdxA* and in *rdxA* and *frxA*; resistant to Mtz at 16 and 64 μg/ml, respectively) are described elsewhere (17). All *H. pylori* strains were cultured under microaerobic conditions on either *Brucella* agar supplemented with 7.5% newborn calf serum (NCS; Sigma) (9, 13) or brain heart infusion (BHI) agar with horse blood (17). *E. coli* strains CC101, CC103, CC104, CC105, and CC106 [*ara*Δ(*lac proB*)/F' *lacI lacZ proAB*$^+$) contain mutations in *lacZ* that render them unable to ferment lactose (4). The *rdxA* gene from *H. pylori* strain 950 was amplified by PCR using previously described *rdxA* oligonucleotide primers (9) and cloned into the pBluescript (pBSK) cloning vector plasmid, creating pGS950. *E. coli* strains expressing RdxA nitroreductase are susceptible to Mtz (10 to 30 μg/ml) when tested on Luria-Bertani (LB) medium (9).

Mtz-induced mutation in *H. pylori*: Rif^r. New mutations to rifampin resistance (Rif^r) which result from changes in *rpoB* (11) were quantified as a measure of Mtz-induced mutation in *H. pylori*. Frozen cultures of isogenic Mtz^s and Mtz^r strains of *H. pylori* (26695, 16R, and 64R) were streaked onto BHI blood agar plates and incubated for 3 days. Individual colonies were respread on fresh medium without Mtz and incubated for 3 days; then 10^7 to 10^8 bacterial cells were spread onto BHI blood agar plates containing various concentration of Mtz (0, 1, 2, and 3 μg/ml for 26695 [wild type]; 0, 3, 8, 16, 25, and 32 μg/ml for 16R; 0, 8, 16, 32, and 90 μg/ml for 64R). Following 3 days of incubation, bacterial cells were suspended in phosphate-buffered saline, and spread on BHI blood agar with rifampin (5 μg/ml); the cells were titered by spotting aliquots of serial 10-fold dilutions on rifampin-free medium. Each test was carried out with six separate single-test cell clones. The data were normalized to 10^8 CFU and expressed as the mean, with *P* values determined by the general linear model (25).

Mtz-induced mutation in *E. coli*: Rif^r. New mutations to Rif^r were quantified as a measure of Mtz-induced mutation in *E. coli* tester strains harboring pGS950 (*rdxA*$^+$) or the parent pBSK vector. *E. coli* strains were grown for 12 h in LB broth containing Mtz at concentrations of 0, 5, 10, and 15 μg/ml, and aliquots were spread on LB agar containing rifampin (25 μg/ml) and on rifampin-free LB agar. The data were normalized to 10^8 bacteria and expressed as the mean of triplicate platings with an error of ±20%.

Mtz-induced mutation in *E. coli*: lac papillation assay of specificity. *E. coli* tester strains (CC101, CC103, CC104, CC105, and CC106) harboring either pGS950 (*rdxA*$^+$) or pBSK control plasmid were grown overnight in LB broth containing ampicillin (100 μg/ml; Boehringer Mannheim) at 37°C. The cultures were standardized to an optical density at 600 nm (OD$_{600}$) of 1.0, and decimal dilutions were prepared in phosphate-buffered saline. Dilutions were plated onto minimal A (MinA) agar medium containing 0.2% glucose, 1 mM MgSO$_4$ · 7H$_2$O, 500 μg of phenyl-β-D-galactoside (Sigma) per ml, and 100 μg of ampicillin per ml (1), supplemented with Mtz at concentrations of 0, 5, 10, and 15 μg/ml. Immediately before use, the plates were spread with 40 μl of X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside; American Bionganics). Total counts were determined by dilution and spreading on MinA (1% glucose) agar plates, which were then incubated for 24 to 36 h. All experiments were repeated three times, all determinations were performed in triplicate in each experiment, and the means and standard deviations were computed. The papillation frequency was determined as the number of blue (β-galactosidase-positive) papillae per total viable count.

Bystander effect. To test for Mtz-induced mutation of bystander cells, a mixture of 10^8 cells of *H. pylori* strain 26695 or its *rdxA* deletion (Mtz^r) derivative 16R plus 10^8 cells of *E. coli* tester strain CC103 at ratios of 1:1, 2:1, and 10:1 was spread on papillation assay plates containing various concentrations of Mtz (0, 5, 10, 15, 32, and 64 μg/ml) as detailed above, except that the plates were incubated under microaerobic conditions. Mtz-induced killing of *E. coli* was determined under microaerobic conditions by dilution at each Mtz concentration (presence or absence of *H. pylori*) by viable count on MinA medium supplemented with 1% glucose. The papillation medium does not support growth of *H. pylori*. In a parallel test for Mtz-induced bystander killing, 10^8 cells of an Mtz^s *H. pylori* strain were mixed with 1,000 *E. coli* cells, and the mixture was spread on a 4-cm^2 area of appropriate agar media (L agar and BHI with serum, plus 0, 10, or 20 μg of Mtz per ml). Incubations were carried out under microaerobic conditions.

Alkaline gel DNA analysis. *H. pylori* strains 26695 (wild type) and 1134 (Mtz^s) and *rdxA* mutant (Mtz^r) derivatives 16R and 1134R, respectively, were grown overnight on *Brucella* agar plates containing 7.5% NCS, collected by centrifugation (7,000 × *g*), suspended in *Brucella* broth, and standardized to an OD$_{600}$ of 0.25. One milliliter of suspension was added to each 500-ml sidearm flask containing 100 ml of *Brucella* broth and NCS. A defined amount of Mtz was then placed in the side arm of the flask. After being incubated statically in a mi-

croaerobic incubator for 6 h, the flasks were sealed and placed on a gyratory shaker at 100 rpm for 16 to 20 h at 37°C. Mtz was then tipped from the side arm into the broth, which was incubated for 30 min with shaking. Aliquots (1 ml) of the cultures were then harvested and prepared for assessment of DNA fragmentation. As a control, 100 μl of cell suspension was treated with 20 mM hydrogen peroxide at 37°C for 15 min to fragment DNA.

E. coli tester strain CC104 harboring either pBSK or its *rdxA*$^+$ derivative pGS950 was grown overnight in MinA broth containing ampicillin (100 μg/ml; Boehringer Mannheim) and 0.5% glucose (1) to a standard OD$_{600}$ of 0.8. One-milliliter aliquots of culture were treated for 30 min at 37°C with Mtz at 0, 10, 20, 40, or 100 μg/ml (final concentration).

Agarose plugs were prepared and treated as described elsewhere (8), with modifications. Briefly, lysozyme (Sigma) and proteinase K (Promega) were added to 100 μl of washed and resuspended cells at a final concentration of 1 mg/ml. Following incubation for 15 min at 37°C, 1.2% molten low-melting-point agarose (Eclipse Molecular Biologicals) and 10% sodium dodecyl sulfate (Sigma) were added to final concentrations of 0.5 and 0.6%, respectively. The mixture was immediately pipetted into plug molds (Bio-Rad Laboratories) and allowed to solidify for 5 to 10 min at 4°C. The agarose plugs were then incubated at 55°C for 16 to 22 h in 2 ml of ESP buffer (pH 9.0), containing 0.5 M EDTA (BDH), 1% (wt/vol) sodium lauryl sarcosine (Sigma), and 1 mg of Proteinase per ml. Following removal of ESP buffer, the plugs were washed 10 min at 55°C with sterile distilled water and four times for 15 min each at 55°C with TE buffer, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA), and then allowed to cool to 4°C.

Alkaline agarose gel electrophoresis generally followed the protocols in reference 31. Briefly, the agarose plugs were placed into the preformed wells of a 0.8% agarose (Vector Biosystems) gel prepared under alkaline conditions (30 mM NaOH, 10 μM EDTA), the wells were sealed in with molten agarose, and then the gel was subjected to electrophoresis for 6 h at 25 mV. The gel was then neutralized for 1 h in 30 mM NaCl–50 mM Tris-HCl (pH 6.0), stained for 1 h with ethidium bromide (0.5 μg/ml; Sigma), destained in distilled water (2 h), and visualized under UV light.

RESULTS

Mtz-induced mutation in *H. pylori*. The ability of Mtz to induce mutation in Mtz^s and in Mtz^r *H. pylori* was scored by measuring frequencies of Rif^r mutants in populations of the normally Rif^s *H. pylori* after 3 days growth on plates containing low, partially inhibitory levels of Mtz. The efficiencies of plating (EOP) of wild-type strain 26695 on medium with 2 and 3 μg of Mtz per ml were about 10^{-2} and 10^{-4}, respectively, of that on Mtz-free medium (EOP = 1.0). Growth on these partially lethal Mtz concentrations increased the Rif^r frequencies among survivors about 6- and 12-fold, respectively (Table 1). In contrast, no significant stimulation of mutation was detected during an equivalent growth period on medium with Mtz at 1 μg/ml, a concentration that did not have any obvious effect on the EOP or colony size of this strain.

The Mtz^r strain 16R (an *rdxA* null deletion mutant derivative of 26695), although fully resistant to 16 μg/ml (EOP = 1.0), was partially killed by higher Mtz concentrations (e.g., EOP = 10^{-2} and 10^{-4} at 25 and 32 μg/ml, respectively). These Mtz concentrations were found to stimulate mutation to Rif^r about 27- and 166-fold, respectively (i.e., even more strongly than had been seen with the wild-type parent grown with Mtz concentrations that were partially lethal to it). In contrast, no such induced mutation was detected in cells treated with lower (subinhibitory) concentrations of Mtz (3, 8, or 16 μg/ml) (Table 1).

Mutant derivatives of strain 16R that are resistant to even higher concentrations of Mtz (e.g., 64 but not 128 μg/ml [strain 64R]) can be obtained by forward mutation at *frxA* (HP0642 in reference 27; an *rdxA* paralog) and other loci (17). Although the basis of this heightened Mtz resistance and the residual Mtz susceptibility of these hyperresistant strains are not fully understood, it is significant that the growth of such strains on medium with Mtz at a partially inhibitory concentration (90 μg/ml) was also mutagenic. Again, no stimulation of mutation was detected after growth with the concentrations of Mtz that were fully tolerated by the strain (8, 16, or 32 μg/ml) (Table 1).

TABLE 1. Mutagenic action of sublethal doses of Mtz on Mtz^r and Mtz^s *H. pylori*

Strain[a]	Mtz dose (μg/ml)	Survival[b]	Rif^r frequency (10^8)[c]	Fold stimulation	P[d]
26695	0	1	12		
1.5R	1	1	14	1.2	NS[e]
	2	10^{-2}	69	6	0.006
	3	10^{-4}	144	12	<0.001
16R	0	1	8		
	3	1	5	0.6	NS
	8	1	12	1.5	NS
	16	1	10	1.3	NS
	25	10^{-2}	213	27	0.0001
	32	10^{-4}	1,328	166	0.0001
64R	0	1	30		
	8	1	36	1.2	NS
	16	1	20	0.7	NS
	32	1	32	1.1	NS
	90	10^{-2}	302	10	0.0002

[a] The strains used were 26695 (wild type), which is Mtz^s (tolerates up to 1.5 μg of Mtz per ml in medium without lethality; 16R, a derivative of 26695 containing an *rdxA*Δ allele in the *rdxA* gene (tolerates up to 16 μg/ml); and 64R, a derivative of 16R containing a point mutation in *frxA* and an additional resistance-enhancing mutation at an unknown locus (tolerates up to 64 μg/ml).

[b] Determined by EOP as described in the text.

[c] Mean frequency of rifampin-resistant mutants in *H. pylori* populations after 3 days of growth on medium containing the indicated concentration of Mtz. Each value is average of six repetitions, each with a different single cell clone.

[d] Calculated using the general linear model (25) by Bill Shannon.

[e] NS, not significantly different from control (growth in Mtz-free medium).

Mtz-induced mutation in *E. coli* expressing *H. pylori* *rdxA*. The ability of Mtz to induce mutation in *E. coli* K-12 that had been rendered Mtz^s by an expressed *H. pylori* *rdxA*$^+$ gene (pGS950) was scored by measuring frequencies of Rif^r mutants after 12 h of growth in broth containing low levels of Mtz. Table 2 shows that growth of *rdxA*$^+$-containing *E. coli* with Mtz at 10 and 15 μg/ml in broth culture decreased viability by 10^{-2} and 10^{-4}, respectively, and increased the Rif^r mutant frequencies ~100- and ~340-fold, respectively. These Mtz concentrations had no effect on viability or Rif^r frequencies in isogenic control strains that did not carry *rdxA*$^+$. Thus, the *H. pylori* RdxA nitroreductase renders *E. coli* highly mutable by Mtz in a dose-dependent fashion.

Specificity of mutation induced by products of Mtz activation. To assess the specificity of mutagenesis following Mtz activation, we scored reversion to Lac$^+$ of a series of isogenic Lac$^-$ strains that differed only in the codon for a critical residue in the active site of β-galactosidase (essentially as in reference 4). Prior studies had indicated that phenotypic reversion from Lac$^-$ to Lac$^+$ occurs by restoration of wild-type sequence in these mutants, not by suppressor mutations elsewhere in *lacZ* or in the genome as a whole. Table 3 shows that growth of these *E. coli* *lac* strains carrying the cloned *rdxA*$^+$

gene on medium devised for quantitative scoring of Lac$^+$ papillae (limiting glucose plus lactose) that was also supplemented with sublethal concentrations of Mtz (5 to 15 μg/ml) resulted in dramatic increases in Lac$^+$ revertant frequencies, whereas no such stimulation was seen with control *E. coli* strains grown in parallel. Certain mutations (CG-to-GC transversions and AT-to-GC transitions) were more strongly stimulated than others (CG to AT and TA to AT). Although reversion of even the least responsive allele (TA to GC) seemed to be stimulated somewhat at the highest usable Mtz concentration (15 μg/ml), this concentration was extremely bactericidal to the *E. coli* (pGS950) tester strains (EOP = 10^{-4}). In consequence, the observed increase in Lac$^+$ papillae in this particular case can be attributed to the effects of bacterial density and the longer period of growth before exhaustion of the limiting glucose, and thus more time for Mtz-induced mutation, rather than a direct mutagenic effect per se on this particular sequence.

Lack of effects on neighboring cells (bystanders). To test whether the mutagenic products of Mtz metabolism by *H. pylori* strains might affect surrounding cells (bystander effect), forward mutation to Lac$^+$ was scored in *E. coli* tester strain CC103 in mixed culture experiments (mixing ratios of *H. pylori* to *E. coli* of 1:1 to 10:1; results at 10:1 presented in Table 4). No Mtz-induced increase in Lac$^+$ reversion frequency or killing of CC103 grown in mixed cultures with either Mtz^s or Mtz^r *H. pylori* was detected. Similarly, under conditions more permissive for the growth of *H. pylori*, there was no detectable Mtz-induced killing of *E. coli* bystanders cocultured with a 10^5-fold excess of Mtz^s *H. pylori* (data not presented). Thus, the mutagenic and bactericidal action of the products of Mtz metabolism seem to be confined primarily to cells in which they are produced, and without impact on neighboring cells.

A control in the bystander mutagenesis experiments, *E. coli* CC103 grown microaerobically without *H. pylori* cells, exhibited Mtz induction of Lac$^+$ reversion in a dose-dependent manner (up to ~30-fold [Table 4]) that was not observed in experiments conducted under aerobic conditions. There was also no decrease in viable counts at any of the Mtz concentrations tested. These findings suggest that under low oxygen tensions, nitroreductases or other redox-active enzymes of *E. coli* activate Mtz, raising the mutation frequency.

DNA fragmentation as a measure of other genetic damage resulting from Mtz activation. To test for DNA damage that might be distinct from Mtz-induced premutagenic lesions, isogenic Mtz^s and Mtz^r *H. pylori* strains were treated for 30 min in *Brucella* broth with different concentrations of Mtz or, for comparison, with 20 mM H$_2$O$_2$, an oxidant that causes DNA fragmentation (31). Figure 1A shows that treatment of the Mtz^s strain 26695 with increasing concentrations of Mtz (5 to 15 μg/ml) caused dose-dependent DNA fragmentation. A similar result was obtained with Mtz^s strain 1134, except that DNA fragmentation was evident at lower Mtz concentrations (Fig.

TABLE 2. Frequency of Rif^r mutants in *E. coli* tester strains

Mtz concn (μg/ml)	Viability[a]	Mutation frequency (no. of Rif^r colonies/10^8 CFU)[b]					Mean ± SD	Fold increase
		CC101	CC103	CC104	CC105	CC106		
0	1	0.47	0.23	0.75	0.94	0.64	0.61 ± 0.27	
10	10^{-2}	83	88	12	96	52	66 ± 34	~100
15	10^{-4}	178	383	118	238	137	210 ± 106	~344

[a] Determined by EOP as described in the text.

[b] Mean of three determinations. All strains contained pGS950 (*rdxA*$^+$), and the data were normalized against data from strains containing pBSK and treated in parallel with Mtz. The bacteria were challenged in LB broth for 12 h in the presence of Mtz as indicated.

5094 SISSON ET AL.

J. BACTERIOL.

TABLE 3. Base specificity of Mtz-induced Lac[+] reversion

Strain	Base Sub. *lacZ*[b]	Papillation frequency[a]							
		pBSK				pGS950 (*rdxA*[+])			
		0	5	10	15	0	5	10	15
E. coli (*lacZ*[+])	GGG AAT **GAG** TCA GGC Glu								
CC101	GGG AAT **T**AG TCA GGC G	1.1	1.2	1.1	1.6	2.5	6 (2.4)	12 (4.8)	115 (46)
CC103	GGG AAT **C**AG TCA GGC G	4.4	5.5	8.5	12.6	4.5	80 (18)	7,407 (1,650)	47,600 (10,578)
CC104	GGG AAT G**C**G TCA GGC A	7.7	5.7	4.4	3.5	9.5	45 (5)	276 (29)	4,555 (479)
CC105	GGG AAT G**T**G TCA GGC A	2.0	1.5	1.5	0.9	1.6	10 (6.3)	77 (48)	2,599 (1,624)
CC106	GGG AAT **A**AG TCA GGC G	2.6	1.0	5.4	6.3	9.0	107 (12)	1,832 (204)	3,222 (358)

[a] Expressed as the number of Lac[+] revertant papillae per 10[8] bacteria plated. The data represent the means of three determinations with an error of <20%. Fold increase is indicated in parentheses for pGS950 results; 0, 5, 10, and 15 indicate Mtz concentration in micrograms per milliliter.
[b] The base substitution required for reversion in each of the LacZ[−] tester strains is depicted in bold.

1C). Analysis of DNA fragmentation on nondenaturing agarose gels indicated that most of the DNA fragmentation observed using alkaline (denaturing) gels resulted from single- rather than double-strand breakage (data not presented). The Mtz[r] strains 16R (Fig. 1B) and 1134R (Fig. 1D) exhibited DNA breakage only at the higher Mtz concentrations that were at least partially lethal to them (e.g., ≥10 μg/ml for 16R and ≥25 μg/ml for 1134R). Since 16R and 1134R contain loss-of-function mutations in *rdxA* (9, 17), additional redox-active enzymes must also act on Mtz at high concentrations, generating products that also contribute to the observed DNA breakage and lethality.

Further demonstration that Mtz activation causes DNA fragmentation was obtained with *E. coli* tester strains carrying a cloned *rdxA*[+] gene. Figure 2 shows that Mtz (5 to 100 μg/ml) caused significant DNA fragmentation when *rdxA*[+] was present (pGS950), whereas no such fragmentation was observed in *E. coli* lacking *rdxA*[+], a strain fully resistant to Mtz.

DISCUSSION

Here we have shown that the potent antimicrobial drug Mtz can be highly mutagenic for *H. pylori* and for *E. coli* strains carrying *rdxA*[+], the *H. pylori* gene whose product is needed for efficient Mtz activation. Mtz is also mutagenic for Mtz[r] strains of *H. pylori* containing null mutations in *rdxA* when the drug is used at concentrations that are considered therapeutic for Mtz[s] strains (i.e., in combination therapies) but only partially toxic for Mtz[r] strains. Products of Mtz activation primarily induce single-strand DNA breakage, with extensive fragmentation occurring at Mtz levels near or higher than the MIC. Reversion tests with *E. coli* *rdxA*[+]-carrying tester strains revealed mostly CG-to-GC transversions and AT-to-GC transitions (with other base substitutions at lower frequencies) with the alleles studied. This suggests the participation of nitro, nitroso, and hydroxylamine intermediates of Mtz, perhaps each differing in reactivity or base specificity (7, 9, 18). Taken together, these studies indicate that Mtz therapy probably induces, as well as selects for, the loss-of-function mutations in *rdxA* that are characteristic of every Mtz[r] clinical isolate tested to date (5, 9, 15–17, 26).

It is striking that Mtz was more mutagenic for Mtz[r] than Mtz[s] *H. pylori* strains (27- to 166-fold versus 6- to 12-fold, respectively) when used at levels that inhibit growth to similar extents. The more efficient mutagenesis in Mtz[r] strains might reflect (i) the high specific activity of RdxA for Mtz (e.g., mutagenic and lethal concentrations are similar), (ii) lower specific activities (substrate specificity for Mtz) of other as yet unknown Mtz-activating enzymes, (iii) changes in the metabolic capacity in response to Mtz (13), and/or (iv) changes in balances of enzymes affecting the fidelity of DNA replication or the efficiencies of repair. That mutagenesis and cell killing were observed in simple *rdxA* mutants illustrates that Mtz can be activated by other cellular enzymes, albeit less efficiently than by RdxA. One such enzyme is FrxA, a flavin nitroreductase of *H. pylori* (9), whose inactivation together with that of

TABLE 4. Bystander effect papillation assay[a]

Mtz concn (μg/ml)	Viability (10[8])	Papillation frequency		
		E. coli	*E. coli* + 26695	*E. coli* + 16R
0	1.20 ± 0.10	2.2 ± 1.3 (1)	4.2 ± 1.6 (1)	1.39 ± 0.9 (1)
5	1.23 ± 0.05	3.8 ± 1.7 (1.7)	7.1 ± 0.9 (1.6)	6.5 ± 0.8 (4.6)
10	1.23 ± 0.11	18 ± 6.3 (9)	11 ± 4.9 (2.6)	16 ± 1.2 (11)
15	1.21 ± 0.12	22 ± 2.2 (10)	15 ± 1.7 (3.6)	21 ± 8 (14)
32	1.10 ± 0.08	37 ± 9.7 (17)	45 ± 14 (11)	48 ± 12 (34)
64	1.25 ± 0.14	54 ± 7.3 (25)	62 ± 5.8 (15)	53 ± 9.9 (38)

[a] Suspensions of *E. coli* CC103 and either *H. pylori* 26695 or its *rdxA*-null mutant derivative 16R were mixed 1:10 and spread on papillation test agar as described in the text. Similar results were obtained with other ratios (e.g., 1:1 and 2:1). Means and standard deviations are from triplicate platings. The reversion to Lac[+] was scored per 10[8] *E. coli* bacteria plated. Viability was determined by dilution of *E. coli* on medium containing each Mtz concentration. The values in parentheses represent the fold increase over that with no Mtz in the medium.
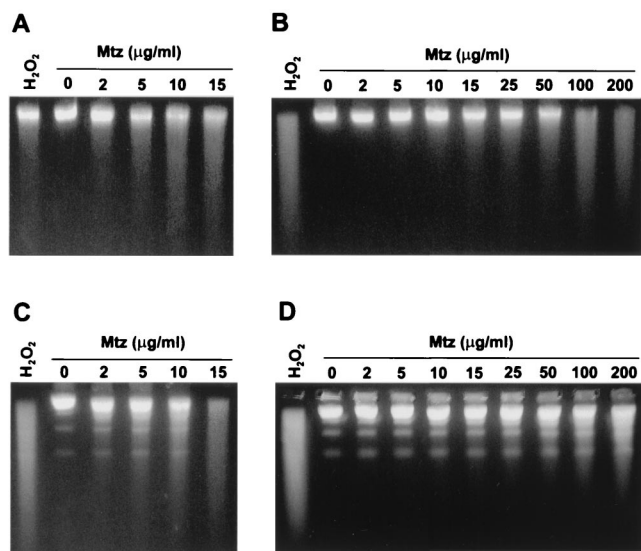
FIG. 1. Mtz-induced DNA fragmentation of MtzR and MtzS strains of *H. pylori*. Mtz^s and Mtz^r strains of *H. pylori* were challenged with various concentrations of Mtz for 30 min as described in the text. The bacteria were suspended and lysed in agarose plugs, and agarose gels were run under alkaline conditions to display the extent of DNA fragmentation of denatured genomic DNA. Bacteria were treated with hydrogen peroxide (20 mM) for 15 min (positive controls). Mtz was used at 0, 2, 5, 10, 15, 25, 50, 100, and 200 μg/ml. (A) *H. pylori* strain 26695 (Mtz^s); (B) its *rdxA* deletion derivative strain 16R (Mtz^r); (C) strain 1134 (Mtz^s); (D) strain 1134R (Mtz^r).

RdxA results in increased Mtz resistance (17). Our finding that Mtz is also mutagenic for hyperresistant (*rdxA frxA*) strains indicates that additional enzymes must also participate to some extent. These additional redox-active enzymes may be quite widespread, since Mtz in high concentration (>150 μg/ml) is toxic for many bacterial species that are considered resistant clinically (7). In general, Mtz toxicity is increased for most bacteria and mammalian cells under hypoxic conditions where nitroreductases, ferredoxins, flavodoxins, cytochrome P450, and nonspecific redox-active components contribute more efficiently to Mtz activation (7, 13, 28). Mutational inactivation of genes encoding these additional Mtz activating enzymes most likely accounts for the stepwise appearance of hyper-Mtz^r mutants of *H. pylori*, although mutations affecting other functions such as DNA repair may also contribute to some extent.

The different efficiencies of Mtz-induced DNA breakage in Mtz^s and Mtz^r strains of *H. pylori* can also be attributed to loss

FIG. 2. Mtz-induced DNA fragmentation of *E. coli* strains carrying *rdxA* of *H. pylori*. *E. coli* strain CC104 containing either pBSK (control) or pGS950 (*rdxA*$^+$) was grown in the presence of Mtz; bacteria were suspended and lysed in agarose plugs and electrophoresed as described in the legend to Fig. 1 and detailed in the text. Hydrogen peroxide was added at 20 mM as a positive control. The distinct bands noted in the various lanes are of plasmid DNA.

of function of the RdxA nitroreductase in Mtz^r strains. Findings that Mtz-induced DNA breakage and mutation were greatest at levels near the MIC in *E. coli* strains expressing *rdxA*$^+$ support this interpretation. In the absence of RdxA activity, *E. coli* is extremely resistant to Mtz (>250 μg/ml), indicating that its own nitroreductases do not contribute appreciably to Mtz activation during growth at normal atmosphere in the concentration range used. However, under microaerobic conditions (6% oxygen), Mtz is more mutagenic for *E. coli* (Tables 3 and 4), suggesting that its nitroreductases or other redox-active enzymes can metabolize this drug. These Mtz-activating enzymes may be induced to higher levels in response to oxygen-limiting conditions (metabolic transition to a more fermentative capacity) or may participate more substantially in removal of reducing equivalents to alternate electron acceptors such as Mtz. Our studies further showed that in hyper-Mtz^r *H. pylori* strains, which contain mutations in a gene(s) in addition to *rdxA* and *frxA*, the efficiency of DNA breakage requires much higher Mtz concentrations. We have shown a correlation between MIC of Mtz for a particular strain and the efficiency of DNA breakage after exposure to Mtz. In this regard, DNA fragmentation was seen at lower Mtz concentrations for strain 26695 16R (*rdxA*Δ), which tolerates 16 μg/ml, than for strain 1134R, which tolerates twice that level (32 μg/ml).

In vitro studies of the interactions of reduced products of Mtz with DNA have indicated that DNA fragmentation is favored over base modification (7). In vivo, Mtz is said to cause more base pair substitution than DNA fragmentation in enteric bacteria (2) but more DNA fragmentation in highly susceptible anaerobic bacteria (7, 28, 29). In one study of selected gene targets, Mtz induced exclusively GC-to-CG transversion mutations in *Bacteroides fragilis* (29). Here we found CG-to-GC transversions and AT-to-GC transitions to be most common in *E. coli* tester strains expressing *rdxA*$^+$, although Mtz induced other substitutions at concentrations that would also cause DNA fragmentation. These base substitution differences are not due to variation among tester strains since these strains showed similar frequencies of mutation to Rif^r. The high frequency of mutation seen at the highest concentrations of Mtz in each of the *E. coli* tester strains may be due to activation of the error-prone SOS repair system, a repair mechanism that is lacking in *H. pylori* (27).

We suggest that Mtz-induced DNA strand breakage, like base substitution mutagenesis, is significant biologically: DNA breakage should stimulate recombination between duplicate and divergent sequences (of which there are many in *H. pylori*) within a given genome and perhaps recombination between different strains as well. Repair of Mtz-induced DNA breaks should also promote the metastable turning on and off of contingency genes, identified by their distinctive repetitive sequences (24), and more generally induce frameshift mutations (21) such as we and others have found after Mtz treatment (9, 15). DNA lesions not involving DNA breakage may be responsible for most of the base substitutions scored here. Mutation and genetic recombination each contribute to the genome diversity that can facilitate adaptation to new and changing conditions in human hosts, the development of resistance to clinically useful drugs, and the evolution of virulence. The emergence of resistance to Mtz, selected and possibly induced by this drug during *H. pylori* infection, has been documented in an animal infection model (16). NH$_2$-containing therapies, in addition to contributing to Mtz hyperresistance, may also induce resistance to other clinically useful drugs (secondary antibiotic resistance). By extrapolation, Mtz therapies might also be mutagenic for the resident intestinal flora (which also live in
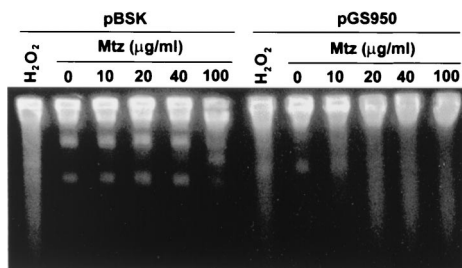
microaerobic and anaerobic environments) and thereby similarly speed the emergence of drug resistance and other evolutionary changes in them.

The biologically active products of Mtz metabolism are considered to be short-lived and thus perhaps unlikely to act on cells other than those in which they are produced (7). It was therefore comforting from a public health perspective that no Mtz-induced killing or mutagenesis of adjacent bystander *E. coli* cells was detected in these experiments. Thus, the products of Mtz metabolism by *H. pylori* probably do not interact synergistically with reactive oxygen and nitrogen metabolites generated in the host inflammatory response that are suspected of contributing to gastric pathologies and cancer (14, 22). As counterpoint, we wish to consider the possibility that a generic function for the nitroreductases studied here is in the catabolism (detoxification) of nitrated aromatic amino acids and other reactive metabolites that are generated during inflammation, and thus of differences between Mtz^r and Mtz^s strains in virulence or in speed of adaptation to host defenses.

REFERENCES

1. Ausubel, F. M., et al. (ed.). 1998. Current protocols in molecular biology, vol. 1, p. 1.1.1. Wiley Interscience, New York, N.Y.
2. Chin, J. B., D. M. K. Sheinin, and A. M. Rauth. 1978. Screening for the mutagenicity of nitro-group containing hypoxic cell radiosensitizers using *Salmonella typhimurium* strains TA100 and TA98. Mutat. Res. 58:1–10.
3. Correa, P. 1996. *Helicobacter pylori* and gastric cancer: state of the art. Epidemiol. Biomarkers Prev. 5:477–481.
4. Cupples, C. G., and J. H. Miller. 1989. A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions. Proc. Natl. Acad. Sci. USA 86:5345–5349.
5. Debets-Ossenkopp, Y. J., R. G. Pot, D. J. van Westerloo, A. Goodwin, C. M. Vandenbroucke-Grauls, D. E. Berg, P. S. Hoffman, and J. G. Kusters. 1999. Insertion of mini-IS605 and deletion of adjacent sequences in the nitroreductase (*rdxA*) gene cause metronidazole resistance in *Helicobacter pylori* NCTC11637. Antimicrob. Agents Chemother. 43:2657–2662.
6. Dunn, B. E., H. Cohen, and M. J. Blaser. 1997. *Helicobacter pylori*. Clin. Microbiol. Rev. 10:720–741.
7. Edwards, D. I. 1993. Nitroimidazole drugs—action and resistance mechanisms. I. Mechanisms of action. J. Antimicrob. Chemother. 31:9–20.
8. Gauton, R. K. 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. J. Clin. Microbiol. 35:2977–2980.
9. Goodwin, A., D. Kersulyte, G. Sisson, S. J. O. Veldhuyzen van Zanten, D. E. Berg, and P. S. Hoffman. 1998. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. Mol. Microbiol. 28:383–393.
10. Glupczynski, Y., and A. Burette. 1990. Drug therapy for *Helicobacter pylori* infection: problems and pitfalls. Am. J. Gastroenterol. 85:1545–1551.
11. Heep, M., D. Beck, E. Bayerdorffer, and N. Lehn. 1999. Rifampin and rifabutin resistance mechanism in *Helicobacter pylori*. Antimicrob. Agents Chemother. 43:1497–1499.
12. Hoffman, P. S. 1999. Antibiotic resistance mechanisms of *Helicobacter pylori*. Can. J. Gastroenterol. 13:243–249.
13. Hoffman, P. S., A. Goodwin, J. Johnsen, K. Magee, and S. J. O. Veldhuyzen van Zanten. 1996. Metabolic activities of metronidazole-sensitive and -resistant strains of *Helicobacter pylori*: repression of pyruvate oxidoreductase and expression of isocitrate lyase activity correlate with resistance. J. Bacteriol. 178:4822–4829.
14. Isaacson, P. G. 1999. Gastric MALT lymphoma: from concept to cure. Ann. Oncol. 10:637–645.
15. Jenks, P. J., R. L. Ferrero, and A. Labinge. 1999. The role of the *rdxA* gene in the evolution of metronidazole resistance in *Helicobacter pylori*. J. Antimicrob. Chemother. 43:753–758.
16. Jenks, P. J., A. Labigne, and R. L. Ferrero. 1999. Exposure to metronidazole in vivo readily induces resistance in *Helicobacter pylori* and reduces the efficacy of eradication therapy in mice. Antimicrob. Agents Chemother. 43:777–781.
17. Jeong, J.-Y., A. K. Mukhopadhyay, D. Dailidiene, Y. Wang, B. Velapatiño, R. H. Gilman, A. J. Parkinson, G. B. Nair, B. C. Y. Wong, S. K. Lam, R. Mistry, I. Segal, Y. Yuan, H. Gao, T. Alarcon, M. L. Brea, Y. Ito, D. Kersulyte, H.-K. Lee, Y. Gong, A. Goodwin, P. S. Hoffman, and D. E. Berg. 2000. Sequential inactivation of *rdxA* (HP0954) and *frxA* (HP0642) nitroreductase genes causes moderate and high-level metronidazole resistance in *Helicobacter pylori*. J. Bacteriol. 182:5082–5090.
18. Lindmark, D. G., and M. Muller. 1976. Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. Antimicrob. Agents Chemother. 10:476–482.
19. Lockerby, D. L., H. R. Rabin, and E. J. Laishley. 1985. Role of the phosphoroclastic reaction of *Clostridium pasteurianum* in the reduction of metronidazole. Antimicrob. Agents Chemother. 27:863–867.
20. Malfertheiner, P., F. Megraud, and C. O'Morain. 1997. Current European concepts in the management of *Helicobacter pylori* infection. The Maastricht consensus report. Gut 41:8–13.
21. McKenzie, G. J., R. S. Harris, P. L. Lee, and S. M. Rosenberg. 2000. The SOS response regulates adaptive mutation. Proc. Natl. Acad. Sci. USA 97:6646–6651.
22. Murakami, K., T. Fujioka, T. Okimoto, Y. Mitsuishi, T. Oda, A. Nishizono, and M. Nasu. 1999. Analysis of p53 gene mutations in *Helicobacter pylori*-associated gastritis mucosa in endoscopic biopsy specimens. Scand. J. Gastroenterol. 34:474–477.
23. Rasmussen, B. A., K. Bush, and F. P. Tally. 1997. Antimicrobial resistance in anaerobes. Clin. Infect. Dis. 24:S110–S120.
24. Saunders, N. J., J. F. Peden, D. W. Hood, and E. R. Moxon. 1998. Simple sequence repeats in the *Helicobacter pylori* genome. Mol. Microbiol. 27:1091–1098.
25. Searle, S. R. 1971. Linear models. J. Wiley & Sons, New York, N.Y.
26. Tankovic, J., D. Lamarque, J.-C. Delchier, C.-J. Soussy, A. Labigne, and P. J. Jenks. 2000. Frequent association between alteration of the *rdxA* gene and metronidazole resistance in French and North African isolates of *Helicobacter pylori*. Antimicrob. Agents Chemother. 44:608–613.
27. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. Ketchum, H. Klenk, S. Gill, B. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. Khalak, A. Glodek, K. McKenney, L. Fitzegerald, N. Lee, M. Adams, E. Hickey, D. Berg, J. Gocayne, T. Utterback, J. Peterson, J. Kelley, M. Cotton, J. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. Hayes, M. Borodovsky, P. Karp, H. Smith, C. Fraser, and J. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature 388:539–547.
28. Townson, S. M., P. F. L. Boreham, P. Upcroft, and J. A. Upcroft. 1994. Resistance to the nitroheterocyclic drugs. Acta Trop. 56:173–194.
29. Trinh, S., and G. Reysset. 1998. Mutagenic action of 5-nitroimidazoles: in vivo induction of GC→CG transversion in two *Bacteroides fragilis* reporter genes. Mutat. Res. 398:55–65.
30. Veldhuyzen van Zanten, S. J. O., P. M. Sherman, and R. H. Hunt. 1997. *Helicobacter pylori*: new developments and treatments. Can. Med. Assoc. J. 156:1565–1574.
31. Zirkle, R. E., and N. R. Krieg. 1996. Development of a method based on alkaline gel electrophoresis for estimation of oxidative damage to DNA in *Escherichia coli*. J. Appl. Bacteriol. 81:133–138.