

Antibiotic resistance mechanisms of *Helicobacter pylori*

Paul S Hoffman PhD

PS Hoffman. Antibiotic resistance mechanisms of *Helicobacter pylori*. *Can J Gastroenterol* 1999;13(3):243-249. Infection with *Helicobacter pylori* is most frequently associated with gastritis and peptic ulcer disease. Antimicrobial intervention, together with proton pump inhibitors, has become the standard therapy for treating this disease. Resistance to clarithromycin and metronidazole, two of the most commonly used antimicrobials for treatment of *H pylori* infections, is often associated with treatment failures and relapse of infection. Clarithromycin resistance arises through mutations leading to base changes in 23S ribosomal RNA subunits, while resistance to metronidazole is due to mutations in the *rdxA* gene, which encodes a novel nitroreductase that is responsible for reductive activation of the drug. Products of metronidazole activation are mutagenic and can be demonstrated to increase both the mutation frequency and the frequency at which antibiotic resistance arises in *H pylori*.

Key Words: Antibiotic resistance, Bismuth, Clarithromycin, *Helicobacter pylori*, Metronidazole, Nitroreductase

Mécanismes de résistance aux antibiotiques de *H. pylori*

RÉSUMÉ : L'infection à *H. pylori* est le plus souvent associée à la gastrite et à l'ulcère gastro-duodéal. L'antibiothérapie et les inhibiteurs de la pompe à protons sont devenus la norme thérapeutique pour le traitement de cette maladie. La résistance à la clarithromycine et au métronidazole, deux des antibiotiques les plus couramment utilisés pour le traitement des infections à *H. pylori*, est souvent en cause dans les échecs thérapeutiques et les récurrences d'infection. La résistance à la clarithromycine résulte de mutations qui entraînent des changements de bases dans les sous-unités 23S de l'ARN ribosomique, alors que la résistance au métronidazole est due à des mutations du gène *rdxA* qui encode une nouvelle nitroréductase responsable d'une baisse de l'activation du médicament. Les produits de l'activation du métronidazole sont mutagènes et on a prouvé qu'ils accroissent la fréquence des mutations et la fréquence à laquelle survient la résistance aux antibiotiques en présence d'*H. pylori*.

Antibiotic resistance is an ever increasing problem associated with the treatment of most microbial infections including *Helicobacter pylori* infections, the major cause of gastritis and peptic ulcer disease. Colonization with *H pylori* leads to a chronic and often lifelong infection in about 60% of the world's population (1-3). Chronic infection with *H pylori* is an accepted risk factor in the development of gastric cancer, one of the most common malignancies worldwide (4). While most *H pylori* infections are asymptomatic and the risk for developing more serious disease is estimated at 20% of infected individuals during a period of 20 years, some investigators believe that *H pylori* infection should be eradicated through antimicrobial intervention or therapeutic vaccination. Any global effort to eradicate *H pylori* with

antibiotics, however, also has to overcome the substantial problem of drug resistance. In examining the mechanisms of antimicrobial resistance of *H pylori*, extrinsic factors must be distinguished from intrinsic ones. Extrinsic factors include extent of infection (microbial load or relative virulence of the strain), immune status of individuals and compliance with treatment regimens. Intrinsic factors include biochemically and genetically based microbial resistance mechanisms. In general, antimicrobial resistance can arise through acquisition of genetic material encoding enzymes that inactivate a particular antibiotic (eg, beta-lactamase, chloramphenicol acetyl transferase or kanamycin phosphotransferase), export antibiotics (eg, tetracycline antiport) or alter the drug

Department of Microbiology and Immunology, Department of Medicine, Division of Infectious Diseases, Dalhousie University, Halifax, Nova Scotia

Correspondence: Department of Microbiology and Immunology, Department of Medicine, Division of Infectious Diseases, Dalhousie University, 7P Sir Charles Tupper Medical Building, Dalhousie University, Halifax, Nova Scotia B3H 4H7. Telephone 902-494-3889, fax 902-494-5125, e-mail hoffmanp@tupdean1.med.dal.ca

target through mutation (eg, penicillin-binding proteins or 23S rRNA in macrolide resistance). To date, no plasmid-borne antibiotic resistance determinants (ie, extrachromosomal resistance transfer factors) or chromosomal resistance determinants have been found in clinical isolates of *H pylori*. Rather, all of the antimicrobial resistance phenotypes described for *H pylori* are mutation based (5-9). The lack of antibiotic resistance genes is borne out by DNA sequence analysis of the entire genome of *H pylori* strains 26695 (10) and J99 (11). While modern molecular biological tools permit establishing a wide range of antimicrobial resistance phenotypes under laboratory conditions (eg, chloramphenicol or kanamycin resistance), the present review focuses on mutation-based resistance mechanisms that are of clinical importance.

ANTIMICROBIAL SUSCEPTIBILITY

In vitro, *H pylori* strains are generally susceptible to penicillins (amoxicillin), cephalosporins, macrolides (clarithromycin), tetracyclines, nitroimidazoles, nitrofurans, furazolidone, nitrothiazoles, quinolones, bismuth salts and even proton pump inhibitors (PPIs) that are commonly employed in combination therapies (12-15). However, monotherapies with these drugs are poorly successful in eradicating infection (3,16,17). The bacterium displays intrinsic resistance to vancomycin, trimethoprim and polymyxin B (1,7,18). However, in vitro susceptibilities do not always correlate with in vivo susceptibilities.

No standard method for determining the antimicrobial susceptibility of *H pylori* strains exists. Variable results are often attributed to poor growth of strains, variations in laboratory technique or simply lack of clear breakpoints (ie, no bimodal distribution) for resistance among the various testing methods (eg, agar dilution, epsilometer test or tube dilution) (1,18). This is particularly true for the assessment of metronidazole susceptibility, which can have a profound effect on therapy. In vitro resistance to metronidazole (greater than 8 µg/mL) correlates with decreased efficiency of therapies containing this drug and often leads to treatment failure (19,20). Frequently, retesting the metronidazole susceptibility of strains (an approach that is not practical in a clinical diagnostic laboratory) can reliably resolve intermediate resistance (4 to 8 µg/mL) to either susceptibility or resistance.

EXTRINSIC FACTORS AFFECTING ANTIBIOTIC BIOACTIVITY

The site of colonization and pathology of *H pylori* infection mitigate against some antibiotics that display in vitro efficacy. In this regard, *H pylori* is a noninvasive pathogen for which the majority of bacteria observed in gastric biopsy material are located in the gastric mucous layer (1). This 'offshore' location may be difficult for antibiotics to reach and together with the constant turnover of the gastric mucous may affect achievement of therapeutic levels. Gastric pH also affects bioactivity and stability of antibiotics (eg, ampicillin). The antimicrobial susceptibility of *H pylori*

may be altered in situ in response to environmental cues that alter the expression of genes that increase resistance to various antimicrobials. For example, acid shock leads to preferential synthesis of several proteins including heat shock (stress) proteins and CagA (1,21).

RECOMMENDED TREATMENTS

Based on the findings of several studies, currently recommended therapies for the eradication of *H pylori* infection often include a PPI (omeprazole, lansoprazole or pantoprazole) together with clarithromycin and either metronidazole or amoxicillin (17,20). Success rates for cure with the use of these combination therapies range from 85% to 95%. However, resistance to metronidazole or clarithromycin results in an increased failure rate of these therapies as well as of therapies containing omeprazole, bismuth salt, metronidazole and tetracycline; bismuth salt, metronidazole and tetracycline; and ranitidine bismuth citrate plus clarithromycin (17,18,20).

Amoxicillin resistance in *H pylori* has been reported at meetings, but other studies have not found resistant isolates from patients (17). The inclusion of a PPI with clarithromycin and metronidazole or clarithromycin and amoxicillin significantly increases the successful eradication of *H pylori*. It has been suggested that the increased pH of the stomach contributes to the increased activity of the antimicrobials against *H pylori* (1,18,20). It is also possible that increased pH leads to differences in gene expression by the bacteria or migration of the bacteria to sites where the antimicrobials are more effective. These areas warrant further investigation.

ANTIBIOTIC RESISTANCE MECHANISMS IN *H PYLORI*

With the recent finding that mutations in a gene encoding an oxygen-insensitive NADPH nitroreductase are responsible for metronidazole resistance of *H pylori* (5), all of the antibiotic resistance mechanisms described so far for *H pylori* can be attributed to mutations in specific genes. That transition base mutations are common in *H pylori* and that these mutations are mostly responsible for drug resistance are themes of the present review. Transition base mutations are purine to purine or pyrimidine to pyrimidine (cytosine → thymine or adenine → guanine) conversions rather than transversion mutations (purine to pyrimidine).

QUINOLONE RESISTANCE

Several studies have suggested that quinolones such as ciprofloxacin can be used in combination therapy to treat *H pylori* infection (6). In *Escherichia coli*, mutations in the DNA gyrase gene (*gyrA*) confer resistance to quinolones (22). A polymerase chain reaction-based analysis of the *gyrA* gene of ciprofloxacin-resistant mutants of *H pylori* revealed mutations in four locations that resulted in amino acid substitutions (6). These included substitutions at amino acid 87 (asparagine → lysine), amino acid 88 (alanine → valine) and amino acid 91 (aspartate →

glycine, asparagine or tyrosine), and a double substitution at amino acids 91 and 97 (alanine → valine). The *gyrA* amplicons from ciprofloxacin-resistant strains readily transformed sensitive strains to resistant ones indicating that these mutations were sufficient to confer resistance to ciprofloxacin. The *H pylori gyrA* gene (10,478 nucleotides) encodes a protein of 826 amino acids that exhibits 52% identity with other bacterial *gyrA* genes and is closely related to the *gyrA* gene of *Campylobacter jejuni* (76.5% identity) (23). Because of the high frequency of resistance, quinolones are not recommended for use in the treatment of *H pylori* infections.

BISMUTH RESISTANCE

Bismuth was one of the early drugs used in triple therapy regimens to treat *H pylori* infection (16). Anecdotal evidence suggested that for some individuals bismuth subsalicylate (BSS) or colloidal bismuth subcitrate (CBS) could effect a cure (16). However, it is estimated that bismuth salts alone have less than a 10% success rate of curing infection. Therefore, they are not recommended as a monotherapy (16,24).

In the laboratory, bismuth salts (BSS and CBS) inhibit the growth of *H pylori* strains, with minimal inhibitory concentration (MIC) values ranging from 4 to 16 µg/mL (19). A systematic series of heterocyclic bismuth sulphur compounds of known chemical structure that exhibit greater in vitro activity against *H pylori* than either CBS or BSS has been developed (25). Such studies should lead to the development of more efficacious bismuth compounds for the treatment of gastrointestinal infections. One advantage of the use of bismuth as an antimicrobial is that the metal is highly toxic to selected bacteria, and resistance has not been demonstrated (19,25). Bismuth, a heavy metal, is highly reactive with the thiol groups of proteins and quickly forms complexes that usually lead to inactivation of enzymatic activity. This is particularly relevant for thiol-containing electron carriers and redox-active compounds found in the membranes of bacteria (26). Preliminary studies in the author's laboratory establish that bismuth complexes become internalized into cytoplasmic inclusions in *H pylori*, as determined by electron microscopic examination of sectioned bacteria (Figure 1) (unpublished data).

CLARITHROMYCIN RESISTANCE

Of the macrolides (erythromycin and azithromycin), clarithromycin is highly effective when used in combination therapy at eradicating *H pylori* infection. As a monotherapy, clarithromycin is only 34% successful (27), and, when metronidazole but not amoxicillin is added, the effectiveness increases up to around 70% (28). Use of PPIs in combination with either metronidazole or amoxicillin increases the effectiveness, with up to 85% to 95% eradication (20).

Resistance to clarithromycin generally parallels the

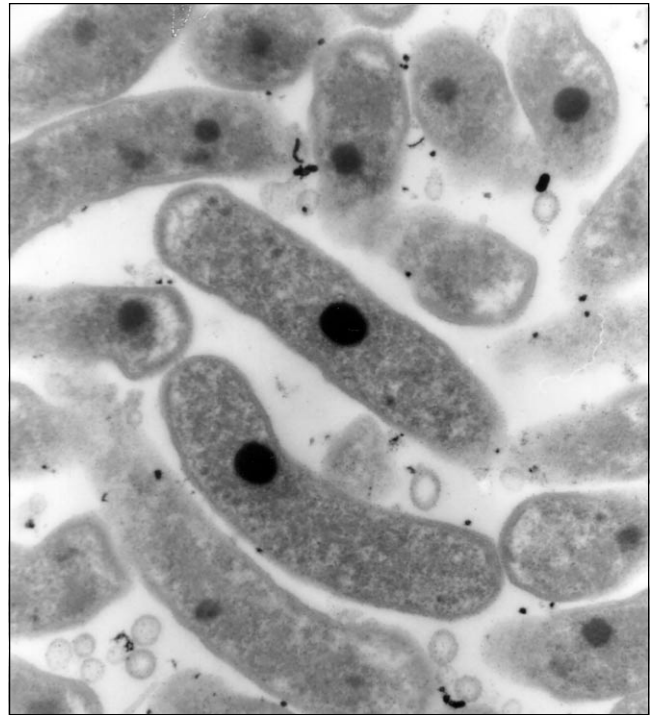


Figure 1) Electron micrograph of an ultrathin section of *Helicobacter pylori* following treatment with a bismuth-containing salt. The large inclusions in the cytoplasm contain bismuth. Original magnification 37, 300x

level of use of macrolides in a particular region. In North America, resistance to clarithromycin is estimated at 1% to 4%, whereas, in France and Belgium, the incidence is as high as 10% (17,18). In Canada, the prevalence is less than 3%. Interestingly, the prevalence of clarithromycin-resistant *H pylori* isolates from individuals treated with the antibiotic but in whom treatment failed to eradicate the infection varied from 20% to 60% (29,30).

Generally, the resistance mechanism for macrolides is similar and involves mutations in the 23S subunit of ribosomal RNA (14). In *E coli*, transition base mutations at positions 2058 (adenine → guanine) and 2059 (adenine → guanine) confer resistance to clarithromycin. In *H pylori*, these positions appear at 2142 and 2143. Several groups have demonstrated that transition base mutations (adenine → guanine) at these positions correlate with clarithromycin resistance (7,8). Work by Taylor et al (7) and confirmed by genomic sequence data indicates that there are two copies of the 23S ribosomal RNA in *H pylori*. In elegant pulsed field gel electrophoresis studies, Taylor et al (7) demonstrated that for most strains of *H pylori*, mutations occur in both 23S rRNA copies. If the mutations are heterozygous, intermediate resistance to clarithromycin is observed. One recent report indicated that a transversion mutation at position 2143 (adenine → cytosine) results in high level resistance to clarithromycin (31). Figure 2 shows the relative position of these substitutions in the V region of the 23S rRNA subunits for *H pylori*. Transition base mutations at position 2142 (adenine → guanine) are more likely to occur in isolates

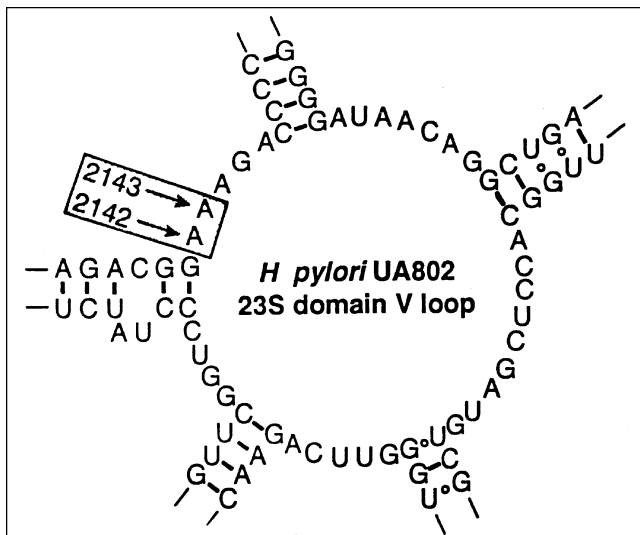


Figure 2) V region of the 23S rRNA subunit of *Helicobacter pylori*. Transition base substitutions at positions 2142 and/or at 2143 are responsible for clarithromycin resistance. Reproduced with permission from reference 7

with MICs exceeding 64 mg/L and to be associated with treatment failure (32).

METRONIDAZOLE RESISTANCE

Metronidazole used in combination with other antibiotics and PPIs is a highly effective therapy against *H pylori* infection (3,18,20). In general, MICs for metronidazole-susceptible (Mtz^S) strains range from 0.1 to 4 $\mu\text{g/mL}$. However, metronidazole-resistant (Mtz^R) strains exhibit MIC values in excess of 32 $\mu\text{g/mL}$ as measured by both the epsilometer test and agar dilution methods. Numerous studies have demonstrated that metronidazole susceptibility status dramatically influences the outcome of treatment (3,17, 20,33). Treatment failure rates as high as 60% have been reported for Mtz^R strains (34).

Metronidazole and other nitroimidazoles are prodrugs because they are biologically inactive until activated by the proper enzyme or target. The basis for toxicity of metronidazole is its conversion through reduction of the 5 nitro-group through a series of four-electron transfers to the DNA-damaging agent hydroxylamine (35). Alkylation of DNA by hydroxylamine and other redox-active intermediates of the reduction pathway cause mostly transition base mutations in DNA that lead to single strand breakage. For example, sublethal concentrations of hydroxylamine hydroxylate guanosine bases (8-hydroxyguanosine) lead to mispairing with thymine, and, following DNA replication, guanosine is replaced with adenosine. Although metronidazole is classified as a mutagen based on the Ames test (35), the drug is generally considered safe when given for a short term (36). The action of metronidazole on anaerobic bacteria and protozoa is due to the reduction of metronidazole by the ferredoxin-linked pyruvate and other ketoacid oxidoreductase enzyme complexes (37). Reduced electron carriers such as ferredoxin or flavodoxin directly reduce

metronidazole. In addition to mutagenic activity on DNA, reduction of metronidazole functions as an electron sink by removing electrons from normal reoxidation routes that ordinarily would lead to ATP production.

In contrast to anaerobic bacteria, where resistance to metronidazole is rare, in microaerophiles such as *H pylori*, Mtz^R is common. In developed countries, Mtz^R of *H pylori* isolates ranges from 10% to 40%, whereas, in developing countries, resistance can range from 50% to 100%. In general, Mtz^R parallels the use of metronidazole for the treatment of unrelated infections in monotherapies that are not sufficient to eradicate *H pylori* infection. This leads to the selection of populations of Mtz^R strains, often in sites that are colonized with Mtz^S variants. Recent studies have shown that isogenic Mtz^S and Mtz^R strains coinhabit the stomach of some individuals (5,38).

Goodwin et al (5) recently reported that mutations in a gene encoding an oxygen-insensitive NADPH nitroreductase are responsible for the Mtz^R phenotype of *H pylori*. The gene *rdxA* was identified by a transformation screen of Mtz^S *H pylori* with cosmids from an Mtz^R strain and the finding of a cosmid clone that conferred resistance at high frequency. *H pylori* strains are able to take up DNA naturally from the environment, and, following recombination, DNA coding for Mtz^R enables the bacterium to grow on media supplemented with metronidazole. Subcloning and DNA sequence analysis identified the *rdxA* gene that encoded the nitroreductase. Cloning and sequencing of the *rdxA* gene of a wild type Mtz^S strain revealed that the mutant *H pylori* strain contained point mutations that led to premature termination of the polypeptide and a biologically inactive product. Allelic exchange mutagenesis achieved through homologous recombination with a chloramphenicol resistance (Cm^R) cassette to disrupt the *rdxA* gene of Mtz^S strain 26695 demonstrated that all colonies selected on the basis of their Cm^R phenotype were also Mtz^R . Complementation of an *rdxA* mutant allele in an Mtz^R strain with the wild type *rdxA* gene provided on a shuttle plasmid that can be maintained in *H pylori* in trans, conferred Mtz^S to the strain. This finding established that expression of wild type *rdxA* (Mtz^S) is a dominant phenotype. Taken together, these results indicate that mutations in *rdxA* are both necessary and sufficient to confer high level metronidazole resistance to *H pylori*.

RdxA is a member of the classical nitroreductases (CNRs) found in many bacterial species; however CNRs are perhaps best studied in *Salmonella typhimurium*, *E coli* and related Gram-negative organisms. Interestingly, it is the CNR of *S typhimurium* that is responsible for reducing nitroaromatic compounds to toxic, mutagenic and carcinogenic compounds in the Ames test (35). Null mutations in the gene encoding CNR render the *S typhimurium* strain resistant to the mutagenic effects of nitroaromatic compounds. A comparison of RdxA with CNR homologues (Table 1) shows amino acid identities of 25% to 30% with similarities approaching 50%. Nitroreductases generally reduce nitroaromatic compounds containing 4 or 5 nitro-

TABLE 1
Similarity of RdxA of *Helicobacter pylori* to other classical nitroreductases

Bacterial species	Protein	Percentage identity	Percentage similarity
<i>Haemophilus influenzae</i>	NfsB	25	48
<i>Enterobacter cloacae</i>	NfnB	30	50
<i>Salmonella typhimurium</i>	Cnr	30	50
<i>Escherichia coli</i>	NfsB	28	49

Data from reference 5

-groups as well as quinones that contain no nitro-group (39).

In general, the CNRs studied from various bacteria exhibit poor specificity for metronidazole. For this reason, facultative anaerobes and aerobes are resistant to metronidazole. What is unique about the nitroreductase of *H pylori* is its ability to use metronidazole at a higher efficiency than what has been found for other CNRs. RdxA differs from other nitroreductases in both isoelectric point (7.9 versus 4.5 for CNRs) and in cysteine content (six residues in RdxA versus one or two for CNRs) (5). Therefore, RdxA may contain an active centre different from that found in CNRs, which may contribute to its lower redox potential and specificity for metronidazole.

Expression of *rdxA* from a high copy plasmid in *E coli* has been shown to render the bacterium susceptible to metronidazole (10 to 30 µg/mL) in contrast to controls exhibiting MIC values in excess of 500 µg/mL (5). By using this assay, where the *rdxA* genes from both Mtz^R and Mtz^S strains were cloned into a high copy plasmid and screened for activity in *E coli*, *rdxA* genes from all of the Mtz^R strains tested were unable to render *E coli* sensitive to metronidazole. In contrast, all *rdxA* genes from Mtz^S strains rendered *E coli* sensitive to metronidazole. Using this assay, Goodwin et al (5) examined matched pairs of *H pylori* strains isolated from a single individual in whom one strain was Mtz^S and the isogenic member of the pair was Mtz^R. In all cases, the Mtz^R *rdxA* amplicon had no effect on the pattern of resistance to metronidazole when cloned into *E coli* (5). In contrast, the *rdxA* allele from each of the Mtz^S members of the pair rendered *E coli* susceptible to metronidazole. When sequenced, the *rdxA* genes from all Mtz^R strains contained transition base mutations that result in amino acid substitutions that render the nitroreductase products inactive (Table 2). These studies established that mutations in *rdxA*, rather than the acquisition of DNA from unrelated strains, are responsible for the Mtz^R phenotype of the strains tested.

TABLE 2
Types of point mutations and amino acid substitutions in matched pairs of metronidazole-resistant and metronidazole-sensitive strains

Strain pair	Mutation				Amino acid substitution
	A → G	C → T	Transversion	Codon*	
H2amt	1			200	Arg → Gly
B1amt	3			47, 143	Tyr → Cys
21cmt	2			50, 63	Gln → Arg
12mtz	1			80	Ala → Thr
10amt3	1	1	1	143	Gly → Val
439/500 [†]	8	15	4		(8 AA changes)

*Codon where base substitution leads to amino acid change; [†]Comparison of divergence in *rdxA* of unrelated *Helicobacter pylori* strains 439 and 500. The number of amino acid changes between these strains is listed. A Adenine; AA Amino acid; Ala Alanine; Arg Arginine; C Cytosine; Cys Cysteine; G Guanine; Gln Glutamine; Gly Glycine; T Thymine; Thr Threonine; Tyr Tyrosine; Val Valine. Data from reference 5

METRONIDAZOLE INCREASES THE MUTATION FREQUENCY AND MAY BE RESPONSIBLE FOR MULTIPLE ANTIBIOTIC RESISTANCE

One unanswered question related to the use of metronidazole for the treatment of a wide range of infections is whether reduction of metronidazole leads to mutations in genes other than *rdxA*, such as those involved in antibiotic resistance. To begin testing this hypothesis, an assay, originally developed by Jeffery Miller (40), was adopted to assess the frequency of transition (adenine → guanine or thymine → cytosine, and vice versa) and transversion base mutations (guanine → thymine). In this assay, mutations are introduced into a common codon (461) of the *lacZ* gene of *E coli* that render the *lac* operon inactive (beta-galactosidase negative). As a result, mutations leading to restoration of the codon can be demonstrated by expression of beta-galactosidase activity. For these studies, *rdxA* was introduced on a plasmid into each of the five tester *E coli* strains containing different base substitutions in codon 461, and then the mutation frequency was assessed for each position following growth in the presence of metronidazole. It was previously shown (5) that the vast majority of mutations in *rdxA* genes are transition base mutations of the kind predicted by the action of hydroxylamine on DNA (41). In contrast, transversion mutations are relatively rare and occur at a low frequency. The frequency of transition base mutations scored was a function of the metronidazole concentration. Concentrations of metronidazole of 5 and 10 µg/mL increased the transition base mutation frequency up to 300-fold. Higher levels of metronidazole were shown to cause a substantial increase in the frequency of transversions (unpublished data), which may be due to a more complex DNA repair and mutator system in *E coli* (40). At 5 to 10 µg/mL of metronidazole, the viability of *E coli* was diminished by 80%, a further indication of the toxicity generated by the

enzymatic conversion of metronidazole to mutagenic products such as hydroxylamine. Hydroxylamine is commonly used to generate transition base mutations. Metronidazole reduction by RdxA also increases the frequency of rifampicin-resistant *E coli* mutants, which are also due to point mutations.

Clarithromycin resistance is more difficult to score in *E coli* strains because, unlike the two copies of the 23S rRNA genes in *H pylori*, *E coli* contains six or more copies. To obtain resistance to clarithromycin, mutations would be required in all copies, a rather rare event. Many studies have examined the effect of sublethal concentrations of antibiotics on the selection of resistant phenotypes (14). However, based on findings that RdxA can reduce metronidazole to toxic and mutagenic products that increase mutation frequency, future experiments should examine *H pylori* strains treated with sublethal concentrations of metronidazole for the development of resistance to clarithromycin. These findings would serve as a model for the potential in vivo development of multiple drug resistance.

Antibiotic resistance of *H pylori* is mutation based and does not involve the acquisition of antibiotic resistance genes from other bacterial species. The correlation between metronidazole use in a community and the incidence of metronidazole resistance in the *H pylori* population in that community can be explained by the action of the NADPH nitroreductase, which activates metronidazole to mutagenic intermediates that introduce transition base substitutions at random into the genome. Because metronidazole is commonly used in monotherapy for the treatment of a wide range of diseases and this monotherapy is not successful in eradicating *H pylori* infection, it is not too surprising that resistant strains arise. Mtz^R status of *H pylori* strains does not alter the fitness of the strains to produce disease. Consequently, Mtz^R siblings are not selected against once metronidazole selection is removed. Whether injudicious treatment with metronidazole leads to mutations in other genes that render a strain resistant to other antibiotics including clarithromycin remains to be determined.

Alternative drugs that ultimately might replace metronidazole include furazolidone and nitazoxanide. The latter compound is a nitrothiazole, which, in preliminary studies, was reported to have an 85% success rate as a monotherapy (unpublished data). Both drugs may have a mechanism of action similar to metronidazole's, though their higher redox potentials may enable these agents to be activated by reductases other than RdxA and, therefore, to show efficacy against Mtz^R strains.

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