Role of the rdxA and frxA genes in oxygen-dependent metronidazole resistance of *Helicobacter pylori*

Monique M. Gerrits, 1 Egbert-Jan van der Wouden, 2 Dorine A. Bax, 1 Anton A. van Zwet, 3 Arnoud H. M. van Vliet, 1 Albertine de Jong, 3 Johannes G. Kusters, 1 Jaap C. Thijs 2 and Ernst J. Kuipers 1

1 Department of Gastroenterology and Hepatology, Erasmus MC-University Medical Center, Rotterdam, The Netherlands
2 Department of Internal Medicine, Bethesda Hospital, Hoogeveen, The Netherlands
3 Regional Public Health Laboratory Groningen/Drenthe, Groningen, The Netherlands

Almost 50 % of all *Helicobacter pylori* isolates are resistant to metronidazole, which reduces the efficacy of metronidazole-containing regimens, but does not make them completely ineffective. This discrepancy between *in vitro* metronidazole resistance and treatment outcome may partially be explained by changes in oxygen pressure in the gastric environment, as metronidazole-resistant (*MtzR*) *H. pylori* isolates become metronidazole-susceptible (*MtzS*) under low oxygen conditions *in vitro*. In *H. pylori* the *rdxA* and *frxA* genes encode reductases which are required for the activation of metronidazole, and inactivation of these genes results in metronidazole resistance. Here the role of inactivating mutations in these genes on the reversibility of metronidazole resistance under low oxygen conditions is established. Clinical *H. pylori* isolates containing mutations resulting in a truncated RdxA and/or FrxA protein were selected and incubated under anaerobic conditions, and the effect of these conditions on the MICs of metronidazole, amoxycillin, clarithromycin and tetracycline, and cell viability were determined. While anaerobiosis had no effect on amoxycillin, clarithromycin and tetracycline resistance, all isolates lost their metronidazole resistance when cultured under anaerobic conditions. This loss of metronidazole resistance also occurred in the presence of the protein synthesis inhibitor chloramphenicol. Thus, factor(s) that activate metronidazole under low oxygen tension are not specifically induced by low oxygen conditions, but are already present under microaerophilic conditions. As there were no significant differences in cell viability between the clinical isolates, it is likely that neither the *rdxA* nor the *frxA* gene participates in the reversibility of metronidazole resistance.

**INTRODUCTION**

*Helicobacter pylori* is a spiral-shaped, Gram-negative bacterium that colonizes the stomach of approximately half the world’s population (Blaser & Berg, 2001). Colonization with *H. pylori* is the most common cause of chronic active gastritis and peptic ulcer disease, and is strongly associated with the development of gastric cancer and gastric lymphoma. Unless treated with antibiotics, *H. pylori* colonization tends to persist for life. Cure of *H. pylori* infection results in ulcer healing and may reduce the risk of gastric cancer and gastric lymphoma development (Sugiyama *et al.*, 2002; Wilhelmsen & Berstad, 1994). *In vitro*, *H. pylori* is susceptible to the majority of antibiotics, but for effective treatment a combination of drugs is required (Debets-Ossenkopp *et al.*, 1999b). Currently used anti-*H. pylori* therapies often consist of two antibiotics with a proton pump inhibitor and/or a bismuth component (Malfertheiner *et al.*, 2002). Metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is a key component of such combination therapies (Malfertheiner *et al.*, 2002; van der Hulst *et al.*, 1996). In Western Europe it has been estimated that 20–45 % of the *H. pylori* isolates are metronidazole-resistant (*MtzR*) (Glupczynski *et al.*, 2001; Lopez-Brea *et al.*, 2001). This percentage is even higher in developing countries and immigrant populations (Falsafi *et al.*, 2004; Loffeld & Fijen, 2003). Although there are conflicting reports concerning the clinical relevance of metronidazole resistance in *H. pylori*, metronidazole resistance reduces the efficacy of metronida-
zole-containing regimens significantly (Jenks et al., 1999a; van der Wouden et al., 1999), but surprisingly does not render them inactive.

Metronidazole is a prodrug that needs to be activated by a reduction of the nitro group that is attached to the imidazole ring. This reduction step leads to the production of DNA-damaging nitroso- and hydroxylamine-compounds. Exposure to these toxic compounds causes DNA damage, and subsequently results in the death of the bacterium. In H. pylori, it is believed that reduction of metronidazole is mainly mediated by an oxygen-insensitive NADPH nitroreductase (RdxA) (Goodwin et al., 1998; Sisson et al., 2002), but recently it has been shown that the NADPH-flavin-oxidoreductase (FrxA) also participates in the reduction of metronidazole (Jeong et al., 2000).

In H. pylori, metronidazole resistance is primarily associated with mutational inactivation of the rdxA gene (Debets-Ossenkopp et al., 1999a; Goodwin et al., 1998; Jenks et al., 1999b). However, recently it has been demonstrated that inactivation of the frxA gene also confers metronidazole resistance, either alone or in association with the rdxA gene (Jeong et al., 2000; Kwon et al., 2000a, b, 2001). Whether mutational inactivation of these two enzymes accounts for metronidazole resistance in all clinical isolates is still being debated (Bereswill et al., 2003; Chisholm & Owen, 2004; Kwon et al., 2000a), but they most likely reflect the two major contributing factors.

The discrepancy between the in vitro resistance to metronidazole and treatment outcome may be explained by the antimicrobial activity of other components in the regimens and/or duration and doses of the therapy (van der Wouden et al., 1999). Apart from these factors, it is likely that low oxygen tension in the gastric environment may also be involved (Smith & Edwards, 1995), since low oxygen conditions affect the activity of metronidazole-reducing enzymes (Jenks & Edwards, 2002). As in vitro MtxK H. pylori isolates become susceptible to metronidazole after a short period of anaerobic incubation (Cederbrant et al., 1992; Smith & Edwards, 1995), it has been suggested that the FrxA protein and/or other ferredoxin and flavin reductases may contribute to the activation of metronidazole under these conditions (Goodwin et al., 1998; Kaihovaara et al., 1998).

In this study the role of null mutations in the rdxA and frxA genes on the reversibility of metronidazole resistance under low oxygen conditions was determined.

**METHODS**

**Strains and growth conditions.** H. pylori isolates used in this study and their respective rdxA and frxA gene status inferred from DNA sequences are listed in Table 1. The H. pylori isolates were routinely grown on Dent plates as described previously (Gerrits et al., 2002b). Broth cultures were grown in Brucella broth supplemented with 3% newborn calf serum (BBN). All cultures were incubated either under microaerophilic (5% O2, 10% CO2 and 85% N2) or anaerobic conditions (10% H2, 5% CO2 and 85% N2) at 37 °C. The anaerobic culture condition was created using the Anoxomat (Mart) in combination with a catalyst. *Escherichia coli* strain DH5α MCR (Life Technologies) was grown on Luria–Bertani agar plates (Sambrook et al., 1989) for 24 h at 37 °C in an aerobic environment. Selection of *E. coli* transformed with pGEM-T Easy clones was performed on LB-agar plates containing ampicillin to a final concentration 100 μg ml⁻¹ (Sigma-Aldrich).

**DNA manipulation.** DNA manipulations were performed according to standard protocols (Sambrook et al., 1989). Oligonucleotides (Table 2; Isogen, PCR-core system 1 (Promega) and pGEM-T Easy vector (Promega) were used according to the manufacturer’s recommendations. Plasmid DNA was isolated with Wizard Plus SV Minipreps DNA Purification System (Promega) according to the manufacturer’s in-

**Table 1.** MtxK H. pylori strains and their rdxA and frxA gene status

<table>
<thead>
<tr>
<th>H. pylori isolate</th>
<th>MIC (μg ml⁻¹)*</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rdxA (HP0954)</td>
<td>frxA (HP0642)</td>
</tr>
<tr>
<td>Truncated rdxA gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH9711-176</td>
<td>24</td>
<td>AY568322</td>
</tr>
<tr>
<td>DM9735-58</td>
<td>&gt;256</td>
<td>AY568328</td>
</tr>
<tr>
<td>DM9716-140</td>
<td>32</td>
<td>AY568326</td>
</tr>
<tr>
<td>Truncated frxA gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH9713-141</td>
<td>&gt;256</td>
<td>AY568323</td>
</tr>
<tr>
<td>DM9642-108</td>
<td>&gt;256</td>
<td>AY568325</td>
</tr>
<tr>
<td>DM9716-140</td>
<td>32</td>
<td>AY568326</td>
</tr>
<tr>
<td>Truncated rdxA and frxA genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BH9714-19</td>
<td>128</td>
<td>AY568324</td>
</tr>
<tr>
<td>DM9727-179</td>
<td>192</td>
<td>AY568327</td>
</tr>
<tr>
<td>ATCC 43504T</td>
<td>&gt;256</td>
<td>Debets-Ossenkopp et al. (1999a) Kwon et al. (2000b)</td>
</tr>
</tbody>
</table>

*MICs shown are means of results from three independent experiments. The isolates were considered to be resistant when the MIC of metronidazole was ≥8 μg ml⁻¹ (Gerrits et al., 2002b).*
Effect of anaerobic incubation on MIC

To evaluate the effect of anaerobic incubation on the MIC of metronidazole, seven MtzR H. pylori clinical isolates and the MtzR H. pylori reference strain ATCC 43504T were selected for this study. To ensure that these strains represented different isolates, RAPD fingerprinting was performed with primers D11344 (Fig. 1), 1254 and D9355 (data not shown). All strains gave different profiles with each of the three primers, indicating they represent unrelated isolates. These seven MtzR isolates and reference strain ATCC 43504T were incubated in microaerophilic and anaerobic conditions, and the MIC of metronidazole was determined by E-test and agar dilution. Under standard microaerophilic culture conditions, the MIC of metronidazole for the eight isolates, as determined by E-test, ranged from 24 to >256 μg ml⁻¹ (Table 3). These MICs for metronidazole decreased under anaerobic conditions. After 4 h of anaerobic incubation, the MIC values for metronidazole dropped below the breakpoint (8 μg ml⁻¹) for three of the eight isolates, and after 8 h, all MtzR isolates had become metronidazole-susceptible (Table 3). In contrast to metronidazole, the MICs for amoxycillin, clarithromycin and tetracycline were stable during anaerobic incubation (data not shown). There were no clear differences found in the MIC values between the E-test and agar dilution.

Effect of metronidazole and anaerobic incubation on cell viability

To determine the effect of metronidazole and anaerobic incubation on cell viability, all seven MtzR H. pylori isolates and the MtzR reference strain ATCC 43504T were cultured in broth under microaerophilic and anaerobic conditions.
Table 3. The effect of anaerobic incubation on metronidazole resistance

MICs shown are means of results from three independent experiments. The isolates were considered to be resistant when the MIC of metronidazole was $\geq 8 \mu g ml^{-1}$ (Gerrits et al., 2002b). T0, time point zero, start point; T2, T4 and T8, after 2, 4 and 8 h of anaerobic incubation, respectively.

<table>
<thead>
<tr>
<th>H. pylori isolates</th>
<th>MIC ($\mu g ml^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0</td>
</tr>
<tr>
<td>Truncated rdxA gene</td>
<td></td>
</tr>
<tr>
<td>BH9711-176</td>
<td>24</td>
</tr>
<tr>
<td>DM9735-58</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Truncated frxA gene</td>
<td></td>
</tr>
<tr>
<td>BH9713-141</td>
<td>&gt;256</td>
</tr>
<tr>
<td>DM9642-108</td>
<td>&gt;256</td>
</tr>
<tr>
<td>DM9716-140</td>
<td>32</td>
</tr>
<tr>
<td>Truncated rdxA and frxA genes</td>
<td></td>
</tr>
<tr>
<td>BH9714-19</td>
<td>128</td>
</tr>
<tr>
<td>DM9727-179</td>
<td>192</td>
</tr>
<tr>
<td>ATCC 43504T</td>
<td>&gt;256</td>
</tr>
</tbody>
</table>

either in the presence or absence of 16 $\mu g$ metronidazole ml$^{-1}$, and at different time intervals the amount of viable bacteria (c.f.u. ml$^{-1}$) was determined. Under standard microaerophilic conditions, the amount of viable cells for all tested MtzR isolates varied between $10^6$ and $10^7$ c.f.u. ml$^{-1}$, and there were no significant differences observed in c.f.u. ml$^{-1}$ between the cultures with and without metronidazole (Fig. 2). Similar data were obtained for the cultures without metronidazole that were incubated anaerobically (Fig. 2). This suggests that neither the incubation with metronidazole nor anaerobic growth conditions alone affect the cell viability of the MtzR isolates.

However, under anaerobic conditions in the presence of metronidazole, the amount of viable cells dropped more than 1000-fold when the MtzR isolates were incubated for 4 h anaerobically, and after 8 h of anaerobic incubation in the presence of metronidazole there were no viable cells present (Fig. 3). Since there were no differences in cell viability or time-course observed between the MtzR isolates containing mutations that resulted in either a truncated RdxA protein or FrxA protein, or a truncation in both, this suggests that neither the rdxA gene nor the frxA gene is involved in the reversibility of metronidazole resistance in H. pylori.

**Effect of de novo protein synthesis on reversibility of metronidazole resistance during anaerobic incubation**

To determine whether H. pylori requires de novo protein synthesis for the reversibility of metronidazole resistance, all experiments were repeated in broth in the presence of the bacterial protein synthesis inhibitor chloramphenicol (10 $\mu g$ ml$^{-1}$). This concentration of chloramphenicol was optimized previously, and its effects on the inhibition of the protein synthesis and cell viability are known (Kusters et al., 1997). When the MtzR isolates were incubated anaerobically in the presence of metronidazole and chloramphenicol, cell viability was reduced (Fig. 4). As there was no significant difference in cell viability between the cultures with and
Implications of experimental data

Metronidazole, a nitroimidazole, is administered as a pro-drug that is activated by the reduction of the nitro group that is attached to an imidazole ring (Edwards, 1986). Since oxygen has a higher reduction potential than metronidazole, this reduction step works out most effectively in an environment with low oxygen tension, such as anaerobic cells and protozoa (Jenks & Edwards, 2002). Surprisingly, the drug was also found to be active against the microaerophilic pathogen *H. pylori* (Lacey et al., 1993). In many strictly anaerobic bacteria, the activation of metronidazole is mediated by the pyruvate : ferredoxin oxidoreductase complex (Smith et al., 1998). In *H. pylori*, this function might be fulfilled by the electron carriers, RdxA (HP0954), FrxA (HP0642), ferredoxin (FdxA, HP0277), flavodoxin (FldA, HP1161), pyruvate : ferredoxin oxidoreductase (PorD, HP1109) and 2-oxoglutarate ferredoxin oxidoreductase (OorD, HP0588). As mutations of the latter four nitroreductases were found to be lethal (Jeong et al., 2000; Kwon et al., 2000a), we only tested the involvement of the *rdxA* and *frxA* genes. In contrast with the findings under normal microaerophilic conditions (Kwon et al., 2000b; Sisson et al., 2002), we showed that neither the *rdxA* nor the *frxA* gene is required for the activation of metronidazole under low oxygen conditions, since strains with one or both genes inactivated still become susceptible to metronidazole under anaerobic conditions.

As Mtz<sup>R</sup> *H. pylori* isolates lose their resistance to metronidazole after exposure to short periods of anaerobiosis in vitro (Cederbrant et al., 1992; Smith & Edwards, 1995), it has been suggested that this reversibility is mediated by compensatory metabolic pathways that are induced under anaerobic conditions (Jenks & Edwards, 2002; Smith & Edwards, 1997). This hypothesis is not supported by our data obtained using the protein synthesis inhibitor chloramphenicol. The loss of metronidazole resistance is mediated by a pre-existing mechanism that functions under anaerobic conditions, and is not dependent on *de novo* protein synthesis when *H. pylori* is exposed to these conditions. Since our data excluded the role of the RdxA and FrxA proteins in this process, we assume that in *H. pylori* metronidazole is reduced by one of the other known nitroreductases.

In summary, Mtz<sup>R</sup> *H. pylori* isolates become fully metronidazole-susceptible at low oxygen conditions, and this does not require *de novo* protein synthesis. This reversibility in metronidazole resistance also occurred in *H. pylori* isolates that contained mutations in the *rdxA* and/or *frxA* genes. Exposure of *H. pylori* to such low oxygen conditions in the gastric mucosa or gastric pit is likely to induce reduction of metronidazole, and thus assist in the eradication of Mtz<sup>R</sup> *H. pylori*.

ACKNOWLEDGEMENTS

The authors would like to thank Andy van Oosterhout for technical assistance.

REFERENCES


