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

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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 (Mtz^R) *H. pylori* isolates become metronidazole-susceptible (Mtz^S) 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.

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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 (Mtz^R) (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-

Abbreviations: Mtz^R, metronidazole-resistant; RAPD, random amplified polymorphic DNA.

The GenBank accession numbers for the *rdxA* and *frxA* gene sequences of seven metronidazole-resistant *H. pylori* strains are AY568322–AY568328 and AY568330–AY568336.

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* Mtz^R *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 % O_2, 10 % CO_2 and 85 % N_2) or anaerobic conditions (10 % H_2, 5 % CO_2 and 85 % N_2) 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 I (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. Mtz^R *H. pylori* strains and their *rdxA* and *frxA* gene status

H. pylori isolate	MIC (μg ml^{-1})*	GenBank accession no.	
		rdxA (HP0954)	*frxA* (HP0642)
Truncated *rdxA* gene			
BH9711-176	24	AY568322	AY568330
DM9735-58	>256	AY568328	AY568336
Truncated *frxA* gene			
BH9713-141	>256	AY568323	AY568331
DM9642-108	>256	AY568325	AY568333
DM9716-140	32	AY568326	AY568334
Truncated *rdxA* and *frxA* genes			
BH9714-19	128	AY568324	AY568332
DM9727-179	192	AY568327	AY568335
ATCC 43504^T	>256	Debets-Ossenkopp *et al.* (1999a)	Kwon *et al.* (2000b)

*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).

Table 2. Oligonucleotides used in this study

Oligonucleotides used for amplification were based on the published genome sequence of *H. pylori* strain 26695 (Tomb *et al.*, 1997).

Gene/primer name	Nucleotide sequence (5′ → 3′)
***rdxA* (HP0954)**	
RdxA-F1	GCTGATTGTGGTTTATGGTTTGG
RdxA-F2	TTGGATCAAGAAAAAAGAAGACAATTATTAA
RdxA-F3	GCTGATTGTGGTTTATGGTTTGG
RdxA-F4	GAGAGCCGGACAGCCAAATG
RdxA-R1	CACCCCTAAAAGAGCGATTAAAACC
RdxA-R2	GCAAGAATGGCGCTCGTT
RdxA-R3	CCCACAGCGATATAGCATTG
***frxA* (HP0642)**	
FrxA-F1	GGATATGGCAGCCGTTTATCATT
FrxA-R1	GAATAGGCATCATTTAAGAGATTA
FrxA-R2	TGGTTCAAGCCCGATTGAAG

structions. Sequencing of the obtained plasmid and PCR products was performed by Baseclear (Leiden, The Netherlands).

Random amplified polymorpic DNA (RAPD) fingerprinting. RAPD analysis of chromosomal DNA was performed with three independent primers, 1254, D11344 and D9355 as described before by Akopyanz *et al.* (1992). RAPD products were separated in 2 % agarose gels containing 0·5 μg ethidium bromide ml^{-1} (Promega).

The influence of the length of anaerobic incubation on antibiotic resistance. MIC values were routinely analysed by E-test (AB Biodisk) (Gerrits *et al.*, 2002b) or agar dilution (Trieber & Taylor, 2002). The plates were incubated under anaerobic conditions for 0, 0·25, 0·5, 1, 2, 4 and 8 h and subsequently incubated for 3 days at 37 °C under microaerophilic conditions, according to the guidelines of the National Committee for Clinical Laboratory Standards. The isolates were considered resistant when the MICs of amoxycillin ⩾8 μg ml^{-1}, clarithromycin ⩾2 μg ml^{-1}, metronidazole ⩾8 μg $ml^{-1}$and tetracycline ⩾4 μg ml^{-1} (Gerrits *et al.*, 2002b). As controls, resistant strains were included for each tested antibiotic (Debets-Ossenkopp *et al.*, 1998; Gerrits *et al.*, 2002a, b). All MIC determinations were performed in triplicate.

The influence of chloramphenicol on metronidazole resistance during anaerobic incubation. Bacteria freshly grown on Dent plates were harvested and inoculated in BBN to a cell density of $\sim 1 \times 10^7$ c.f.u. ml^{-1}, and incubated overnight with gentle agitation under microaerophilic conditions. Fumaric acid (Sigma-Aldrich, final concentration 0·1 %) was then added to the overnight culture to facilitate the generation of anaerobic conditions (Jenks *et al.*, 1999b). The culture was then split into 10 ml portions and when indicated supplemented with metronidazole (Sigma-Aldrich, final concentration 16 μg ml^{-1}) and/or chloramphenicol (Sigma-Aldrich, final concentration 10 μg ml^{-1}). Subsequently, the cultures were incubated under microaerophilic or anaerobic conditions for 0, 2, 4, 6 and 8 h. At each time point 1 ml of the broth was taken and washed twice with PBS to remove the antibiotics. In order to determine the number of viable bacteria, 50 μl of undiluted suspension and 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated on Columbia agar plates containing 7 % lysed horse blood and incubated under microaerophilic conditions. When present, colonies were counted and data are expressed as c.f.u. ml^{-1}.

RESULTS AND DISCUSSION

Effect of anaerobic incubation on MIC

To evaluate the effect of anaerobic incubation on the MIC of metronidazole, seven Mtz^R *H. pylori* clinical isolates and the Mtz^R *H. pylori* reference strain ATCC 43504^T 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 Mtz^R isolates and reference strain ATCC 43504^T 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 Mtz^R 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 Mtz^R *H. pylori* isolates and the Mtz^R reference strain ATCC 43504^T were cultured in broth under microaerophilic and anaerobic conditions

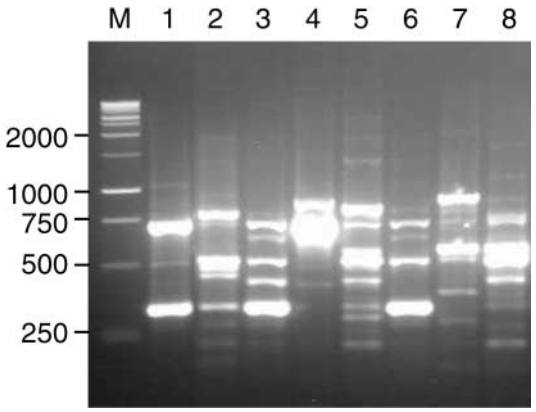

Fig. 1. The *H. pylori* isolates used in this study represent unrelated isolates, as shown by RAPD-PCR. DNA isolated from the Mtz^R isolates was amplified with primer D11344 according to standard procedures (Akopyanz *et al.*, 1992). The RAPD-PCR products were separated on a 2·0 % agarose gel and stained with ethidium bromide. Lane 1, *H. pylori* reference strain ATCC 43504^T, lanes 2–8, BH9711-176, BH9713-141, BH9714-19, DM9735-58, DM9727-179, DM9642-108 and DM9716-140, respectively. M, 1 kb marker (Promega).

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 ⩾8 μg ml^{-1} (Gerrits *et al.*, 2002b). T_0, time point zero, start point; T_2, T_4 and T_8, after 2, 4 and 8 h of anaerobic incubation, respectively.

H. pylori isolates	MIC (μg ml^{-1})			
	T_0	T_2	T_4	T_8
Truncated *rdxA* gene				
BH9711-176	24	16	12	3
DM9735-58	>256	32	4	0·75
Truncated *frxA* gene				
BH9713-141	>256	48	24	3
DM9642-108	>256	64	24	2
DM9716-140	32	8	4	0·75
Truncated *rdxA* and *frxA* genes				
BH9714-19	128	48	12	4
DM9727-179	192	96	16	0·5
ATCC 43504^T	>256	48	6	0·25

either in the presence or absence of 16 μg 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 Mtz^R 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 Mtz^R isolates.

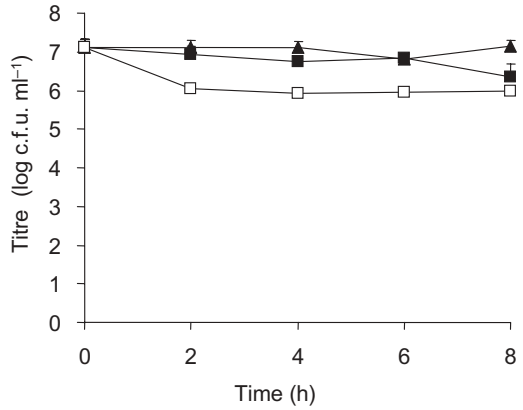

Fig. 2. The influence of metronidazole and anaerobic incubation on cell viability. *H. pylori* was grown microaerophilically in the presence (▲) and absence (■) of 16 μg metronidazole ml^{-1}, and anaerobically (□). At different time points the c.f.u. ml^{-1} was determined. Results shown are a representative example of one of the Mtz^R isolates, BH9714-19, and are the means (±SD) of two independent experiments performed in duplicate.

However, under anaerobic conditions in the presence of metronidazole, the amount of viable cells dropped more than 1000-fold when the Mtz^R 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 Mtz^R 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 μg 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 Mtz^R 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

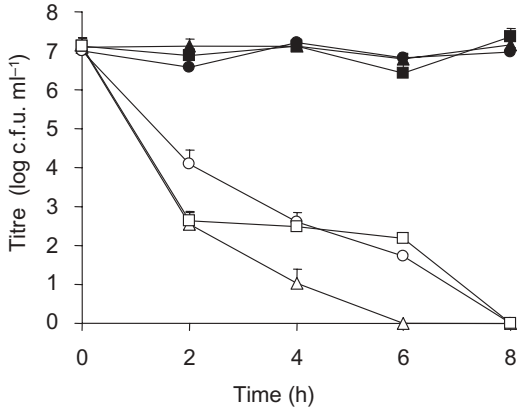

Fig. 3. Involvement of the *rdxA* and/or *frxA* gene in the reversibility of metronidazole resistance under anaerobic conditions. *H. pylori* was grown in microaerophilic (filled symbols) and anaerobic (open symbols) conditions in the presence of 16 μg metronidazole ml^{-1}, and the c.f.u. ml^{-1} were determined. Results shown are for representative examples of the Mtz^R isolates tested. ■/□, BH9714-19 (containing mutations resulting in truncated RdxA and FrxA proteins); ▲/△, DM9735-58 (containing mutations resulting in a truncated RdxA); ●/○, BH9713-141 (containing mutations resulting in a truncated FrxA). Results shown are the means (±SD) of two independent experiments performed in duplicate.

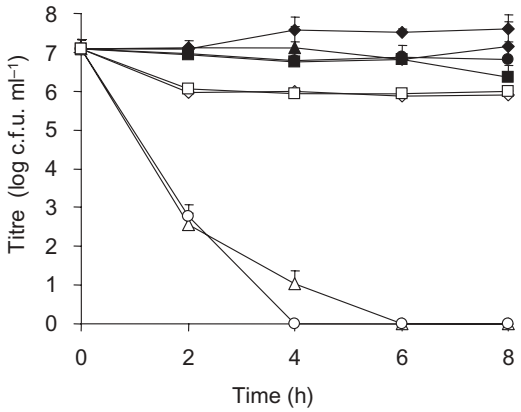

Fig. 4. The effect of blocking *de novo* protein synthesis on the reversibility of metronidazole resistance. *H. pylori* was grown in broth either with or without metronidazole (16 μg ml^{-1}) and/or chloramphenicol (10 μg ml^{-1}) under microaerophilic (filled symbols) or anaerobic (open symbols) conditions. Results shown are for a representative example (strain BH9714-19) of the Mtz^R isolates tested. ■/□, Control; ▲/△, with metronidazole; ◆/◇, with chloramphenicol; ●/○, with metronidazole and chloramphenicol. Results shown are the means (±SD) of two independent experiments performed in duplicate.

without chloramphenicol these results indicate that factors that are involved in the reversibility of metronidazole resistance are already present under microaerophilic conditions. *De novo* protein synthesis is not required for this phenomenon.

Implications of experimental data

Metronidazole, a nitroimidazole, is administered as a prodrug 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^R *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^R *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^R *H. pylori*.

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