Frameshift mutations in frxA occur frequently and do not provide a reliable marker for metronidazole resistance in UK isolates of *Helicobacter pylori*

Stephanie A. Chisholm and Robert J. Owen

*Helicobacter pylori* infection is most often associated with asymptomatic gastritis, but it can lead to the development of severe conditions including peptic ulcer disease (PUD) (Anonymous, 1994), lymphoproliferative disorders and early stage gastric carcinoma (Helicobacter and Cancer Collaborative Group, 2001). Treatment with current triple therapy regimes can facilitate healing of duodenal ulcers and prevents relapse of PUD, but antibiotic resistance is now a major contributing factor in treatment failure (Dore et al., 2000; van der Wouden et al., 1999). Rates of metronidazole (Mtz) resistance are approximately 30% in western Europe (Megraud et al., 1999) but can be as high as 90% in developing countries where Mtz is a widely used therapeutic agent for parasitic infections (Alarcon et al., 1999). Surveillance of Mtz resistance is problematic as there are no standardized protocols for culture-based susceptibility testing. The possibility of a simple molecular test for Mtz resistance analogous to those described for clarithromycin susceptibility testing (Chisholm et al., 2001; Maeda et al., 2000; Matsumura et al., 2001; Trebesius et al., 2000) has been hindered by a lack of understanding of the precise mechanism of Mtz action and of resistance development in *H. pylori* (Mendz & Megraud, 2002; Jenks & Edwards, 2002).

Although there is substantial evidence in support of a role for the oxygen-insensitive nitroreductase (RdxA) protein in Mtz resistance, the occurrence of Mtz-resistant (Mtz\(^R\)) strains that possess an apparently wild-type *rdxA* gene is well documented (Chisholm & Owen, 2003; Goodwin et al., 1998; Jenks et al., 1999; Kwon et al., 2001a; Tankovic et al., 2000; Wang et al., 2001). Inactivation of the *frxA* gene that encodes NAD(P)H flavin oxidoreductase was recently shown to increase the minimum inhibitory concentration (MIC) of Mtz-sensitive (Mtz\(^S\)) strains to resistant levels, while dual inactivation in combination with *rdxA* results in even higher MICs. In addition, inactivated *frxA* genes from clinical isolates can transform *H. pylori* from a Mtz\(^S\) to a Mtz\(^R\) resistotype (Kwon et al., 2000a). In contrast, there is evidence that *frxA* inactivation alone is insufficient to confer a Mtz\(^R\) phenotype, but it can raise the Mtz MIC in *rdxA*-deficient mutants (Jeong et al., 2000). Further investigations suggest that *frxA* inactivation may slow bacterial killing by Mtz but not cause resistance and that two types of *H. pylori* exist: Type I, where resistance can develop by mutation in *rdxA* only, and Type II, which requires dual mutation of both *rdxA* and *frxA* for a Mtz\(^R\) phenotype (Jeong et al., 2001). However, while a survey of 12 clinical isolate pairs confirmed that high-level resistance is linked to mutations in both *rdxA* and *frxA* (Kwon et al., 2001a), this and one other study examining clinical isolates demonstrated that intermediate or low-level resistance could occur in isolates containing mutated *frxA*.

**INTRODUCTION**

*Helicobacter pylori* infection is most often associated with asymptomatic gastritis, but it can lead to the development of severe conditions including peptic ulcer disease (PUD) (Anonymous, 1994), lymphoproliferative disorders and early stage gastric carcinoma (Helicobacter and Cancer Collaborative Group, 2001). Treatment with current triple therapy regimes can facilitate healing of duodenal ulcers and prevents relapse of PUD, but antibiotic resistance is now a major contributing factor in treatment failure (Dore et al., 2000; van der Wouden et al., 1999). Rates of metronidazole (Mtz) resistance are approximately 30% in western Europe (Megraud et al., 1999) but can be as high as 90% in developing countries where Mtz is a widely used therapeutic agent for parasitic infections (Alarcon et al., 1999). Surveillance of Mtz resistance is problematic as there are no standardized protocols for culture-based susceptibility testing. The possibility of a simple molecular test for Mtz resistance analogous to those described for clarithromycin susceptibility testing (Chisholm et al., 2001; Maeda et al., 2000; Matsumura et al., 2001; Trebesius et al., 2000) has been hindered by a lack of understanding of the precise mechanism of Mtz action and of resistance development in *H. pylori* (Mendz & Megraud, 2002; Jenks & Edwards, 2002).

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only (Kwon et al., 2001a; Marais et al., 2003). Thus, the exact contribution of \(frxA\) inactivation to Mzr resistance remains controversial.

In the present study, \(frxA\) sequences were examined in a unique collection of clinical isolates recovered from English dyspeptic patients that had been examined previously for mutations in \(rdxA\) (Chisholm & Owen, 2003). The study was extended to evaluate the prevalence and significance of an adenine deletion at nucleotide 53 by the development and application of a novel real-time PCR screening assay using the LightCycler instrument. Specific aims were to examine the contribution of \(frxA\) mutation to Mzr resistance in a larger collection of clinical isolates than has been examined to date, to determine the frequency of early frameshift mutations in \(frxA\) and to assess the significance of these in terms of \textit{in vitro} Mzr susceptibility based on the E-test.

**METHODS**

**Bacterial cultures.** The study examined a collection of 44 isolates of \(H. pylori\) collected from 18 dyspeptic patients (A–R) as described previously (Chisholm & Owen, 2003). Isolates were obtained from 11 patients from Ipswich (A–G) and London (H–K) before and after they had received Mtx-containing therapy. Our previous characterization of the Mtx MIC by E-test demonstrated that 7/11 isolates were mixed Mtx\(^{3}\)/Mtx\(^{8}\) before treatment and Mtx\(^{8}\) post-treatment (patients A, B, E, G, H, I, K). 3/11 isolates were Mtx\(^{5}\) before therapy and Mtx\(^{8}\) after (patients C, F, J) and 1/11 was Mtx\(^{3}\) pre-treatment and mixed Mtx\(^{2}\)/Mtx\(^{8}\) post-treatment (patient D). All mixed infections had been purified into separate Mtx\(^{5}\) and Mtx\(^{8}\) populations and shown to be phenotypic strain variants by amplified fragment length polymorphism (AFLP) (Chisholm & Owen, 2003). As this was the first study to investigate \(frxA\) mutation in isolates from the UK, we also examined mixed Mtx\(^{2}\) and Mtx\(^{8}\) subpopulations recovered pre-treatment either from the antrum alone (patients L–N) or from the antrum and the corpus (patients O–R) of dyspeptic patients in London (Chisholm & Owen, 2003).

In addition, \(H. pylori\) isolated pre-treatment from antral gastric biopsies of 119 dyspeptic patients who underwent endoscopy in London \((n = 81)\), Bangor, North Wales \((n = 26)\), Leeds, northern England \((n = 7)\), Chelmsford, south-eastern England \((n = 3)\) and Portsmouth, southern England \((n = 2)\), were tested for a frameshift mutation at nucleotide 53 by a novel real-time assay. Isolate resistotypes had been determined previously by E-test as either Mtx\(^{5}\) \((n = 61)\) or Mtx\(^{8}\) \((n = 58)\) (N. C. Elviss, personal communication).

**DNA extraction.** Bacterial genomic DNA was extracted from all cultures following the CTAB method described by Wilson (1987). Extracted DNA was stored \((-20^\circ\text{C})\) until required.

**Amplification and sequencing of \(frxA\).** Two overlapping fragments of \(frxA\) were amplified from most strains by using primer pairs EFR-1/ BFR-3 and EFR-2/EFR-4 (Kwon et al., 2001a). Where amplification failed due to strain sequence variation, published primers FrxET/FrxER (Jeong et al., 2001) and novel primers FrxA2F \((5'-AGG TTC GCT CAA ATC ATC A-3')\) and FrxA2R \((5'-TTC AAT CAC TTC ATA AAT AAC-3')\) were also used to generate \(frxA\) fragments. Briefly, fragments were amplified in a 100 \(\mu\text{l}\) reaction containing 200 ng DNA from culture, 200 \(\mu\text{M}\) (each) dNTP (Invitrogen), 0.4–8 \(\mu\text{M}\) each of the appropriate primers (MWG Biotech), 2.0 mM MgCl\(_2\), 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 0.2–2% (v/v) glycerol, 2 \(\mu\text{M}\) Taq polymerase (Invitrogen). Reactions were incubated in a DNA Engine (MJ Research, Genetic Research Instrumentation) thermal cycler for 5 min at a denaturation temperature of 95 \(^\circ\text{C}\), followed by 35 cycles of denaturation at 95 \(^\circ\text{C}\) for 30 s, annealing at 48 \(^\circ\text{C}\) for 30 s and elongation at 72 \(^\circ\text{C}\) for 1 min, followed by 5 min at 72 \(^\circ\text{C}\). Gene sequences were determined as described (Owen & Xerry, 2003) and sequence chromatograms were examined in CHROMAS version 1.42 (Griffith University, Australia). Corrected sequences were aligned in GENEBASE version 1 (Applied Maths). All novel sequences were also aligned with the 23 \(frxA\) sequences currently held in GenBank.

**Detection of the \(frxA\) frameshift mutation at nucleotide position 53.** Multiple alignment of \(frxA\) sequences determined in the course of this study and the 23 \(frxA\) sequences held in GenBank enabled design of novel assay FS-53, to detect a frameshift mutation in \(frxA\) caused by deletion of adenine 53. Primers targeted conserved regions of \(frxA\) that flanked nucleotide 53, while a labelled probe (FS-53Pr) was designed that was exactly complementary to the mutated sequence, spanning six adenine residues instead of the seven found in wild-type strains. A 265 bp fragment of \(frxA\) containing nucleotide 53 was amplified and the resultant PCR product was screened for FS-53 by using the LightCycler instrument (Roche Diagnostics) in a 20 \(\mu\text{l}\) reaction containing 20 ng DNA, 1 \(\times\) FastStart DNA Master SYBR Green 1 master mix (Roche Diagnostics), 6 mM MgCl\(_2\), 0.5 \(\mu\text{M}\) each primer, EFR-1 andod (5'-TCT CAA GCG GAA AAA TCG-3') and frxRFS (5'-ATC TTC TTT CAT CGG TTC A-3') (MWG Biotech), 5 \(\mu\text{M}\) labelled probe FS-53Pr (5'-LC Red 640-ATT TGC TGC AAA AAA TAC GAT C-P-3') (TIB MOLBIOL). Amplification reactions were performed, following a 10 min incubation (95 \(^\circ\text{C}\)), by 50 cycles of denaturation (95 \(^\circ\text{C}\) for 0 s), annealing (45–48 \(^\circ\text{C}\) for 0 s, temperature increment 4 \(^\circ\text{C} \cdot \text{s}^{-1}\)) and extension (72 \(^\circ\text{C}\) for 5 s). Amplicon generation was monitored by measuring SYBR Green 1 fluorescence (Channel F1) after each extension stage. Probe hybridization melting-point analysis was performed by continuous measurement of LC Red 640 dye fluorescence (Channel F2) over the temperature range 45–95 \(^\circ\text{C}\) (temperature increment 0.1 \(^\circ\text{C} \cdot \text{s}^{-1}\)).

**RESULTS AND DISCUSSION**

**Comparison of translated FrxA amino acid sequences in Mtx\(^{5}\) and Mtx\(^{8}\) strains**

The aim of this study was to investigate the potential contribution of naturally occurring \(frxA\) mutations to Mzr resistance in \(H. pylori\) by conducting the largest survey of clinical isolates described to date. Our strategy was, firstly, to sequence \(frxA\) genes from paired UK isolates, recovered before and after eradication therapy, and also for mixed MtxR and MtxS subpopulations, usually recovered pre-treatment, to establish if either mutations observed post-treatment were also present pre-treatment in the matched MtxR subpopulation, or if \textit{de novo} mutation had occurred as a consequence of eradication therapy.

In 14/18 (77.7%) patient sets examined, no mutational differences were observed in any of the matched populations, while frameshift mutations were observed only in MtxR populations in three patients (C, I and J) and only in the MtxS strain in patient Q (Table 1). A multiple alignment of FrxA sequences with 23 sequences of other isolates held in GenBank demonstrated that at least one strain from 17/18 (94.4%) patient sets had a frameshift mutation that led to premature truncation of the FrxA protein (Table 1, Fig. 1). In 13/18 patient sets, frameshift mutations were observed in all
strains, regardless of Mtz resistotype. Frameshifts occurred at nucleotide 53 in 11/17 (64.7%) patient sets that were mutated, usually due to a single adenine deletion. In most cases this led to early protein truncation at codon 39 (Fig. 1), with the exception of patient L where a 2 bp (AA) deletion was observed at position 53 and also patient N where a G117T substitution altered codon 39 (Fig. 1). Frameshift and missense mutations have been reported in previous investigations that examined frxA of two MtzS and four MtzR strains (Kwon et al., 2000a) and a total of 21 paired isolates (Kwon et al., 2001a; Marais et al., 2003), but these were observed in MtzR strains only (Marais et al., 2003). In contrast, our study demonstrated that such mutations also occur in MtzS strains. The differences between our results and those reported previously may either be attributable to geographical variations in frxA or our significantly larger study population allowed more representative characterization of this gene.

Previous reports have suggested that mutated frxA may contribute to high-level resistance only if combined with mutated rdxA (Jeong et al., 2000, 2001). Examination of frxA in our strain set in relation to the MICs and rdxA sequences determined previously (Chisholm & Owen, 2003) demonstrated that mutated frxA gene sequences were present in seven isolate sets (patients A, E, I, K, O, P, R) that displayed high-level resistance (MIC > 256 mg l⁻¹) but had no mutations in rdxA. This finding shows that high-level resistance can occur in isolates with apparently unaltered rdxA. However, as the frxA mutations were also observed in MtzS strains, we conclude that they are unlikely to contribute to the resistance of these isolates.

### Distribution of the frxA FS-53 mutations amongst 119 isolates

Frameshift of frxA due to a single adenine deletion at nucleotide 53 was the most frequently observed mutation, in 11/18 patients. As the number of isolate sets investigated by sequencing was comparatively small (n = 18), a novel PCR-based probe hybridization melting-point analysis assay (FS-53) was developed to allow rapid screening of a larger, more representative, number of MtzS and MtzR strains of *H. pylori*. Validation of assay FS-53 on 44 isolates of known frxA sequence (patients A–R) demonstrated that it allowed easy, accurate and rapid identification of strains containing the deletion mutation. Strain sequences with a single adenine deletion at nucleotide 53, containing a run of six adenine residues rather than the seven found for the wild-type gene, were exactly complementary to probe FS-53Pr and so generated a melting peak indicating a probe–template dissociation temperature of approximately 61 °C. Wild-type strain sequences that retained seven adenines were mismatched with the probe and generated a melting curve indicative of a lower dissociation temperature of approximately 59 °C (Fig. 2),

### Table 1. Sequence variations in *H. pylori* frxA identified by comparison of matched MtzS and MtzR strains recovered from patients before and after therapy or simultaneously as a mixed infection

<table>
<thead>
<tr>
<th>FrxA mutation (nucleotide position)</th>
<th>FrxA amino acid sequence change (codon)</th>
<th>Patient</th>
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<tbody>
<tr>
<td>Pre-treatment</td>
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<tr>
<td>MtzS</td>
<td>MtzR</td>
<td>MtzR</td>
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<tr>
<td>Frameshift (53)</td>
<td>Frameshift (53)</td>
<td>Stop codon (39)</td>
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<td>Frameshift (53)</td>
<td>Framesshift (53)</td>
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<tr>
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<td>Frameshift (211)</td>
<td>Stop codon (91)</td>
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<td>Frameshift (209)</td>
<td>Stop codon (74)</td>
</tr>
<tr>
<td>No mutation</td>
<td>NA†</td>
<td>Stop codon (21)</td>
</tr>
<tr>
<td>No mutation</td>
<td>NA†</td>
<td>None</td>
</tr>
<tr>
<td>Frameshift (53)</td>
<td>Frameshift (53)</td>
<td>NA‡</td>
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<tr>
<td>Frameshift (24)</td>
<td>Frameshift (24)</td>
<td>No mutation</td>
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<tr>
<td>Frameshift (53)</td>
<td>Frameshift (53)</td>
<td>NA‡</td>
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<tr>
<td>Frameshift (53)</td>
<td>No mutation</td>
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<tr>
<td>Frameshift (24)</td>
<td>No mutation</td>
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<tr>
<td>Frameshift (24)</td>
<td>No mutation</td>
<td>Stop codon (39)</td>
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</tbody>
</table>

*Not applicable as post-treatment strains were different by amplified fragment length polymorphism (AFLP) genotyping (Chisholm & Owen, 2003).
†Not applicable as mixed susceptibility infections were not observed.
‡No post-treatment isolates were available.
§Not applicable as only MtzR strains were observed pre-treatment.
while the probe failed to hybridize with sequences where the first of the seven adenine residues had been replaced with a guanine.

A potential limitation of any probe hybridization melting temperature-based analysis is that other mutations not associated with the polymorphism under investigation could lower the probe dissociation temperature. In all 44 sequenced isolates examined, all lower melting temperatures were due to absence of mutation FS-53. This screening assay was developed principally to determine if mutation FS-53 was found in MtzS isolates of *H. pylori* in the general pre-treatment dyspeptic population. Application of assay FS-53 to 119 isolates generated melting peaks identical to those of the mutation-positive controls in 24 isolates, which were defined as containing the FS-53 mutation. This approach demonstrated that the pre-treatment prevalence of adenine deletion at position 53.

We infer from the results of this study that inactivation of frxA alone by mutation does not inevitably lead to Mtz resistance in *H. pylori*. This is in agreement with a previous
study that demonstrated inactivated frxA genes did not always transform H. pylori phenotype from MtzS to MtzR (Jeong et al., 2000). Furthermore, purified recombinant FrxA protein did not reduce Mtz even though Escherichia coli could be transformed with frxA to become more sensitive to Mtz, thereby providing evidence that FrxA does not naturally play a role in Mtz action and in resistance development (Sisson et al., 2002). Previous transformation-based studies and construction of knockout mutants have suggested that frxA inactivation can lead to resistance development (Jeong et al., 2000, 2001; Kwon et al., 2000a, b, 2001b). However, as our results suggest that inactivation of frxA leading to protein truncation occurs frequently and does not necessarily lead to Mtz resistance, FrxA may be a non-essential enzyme. It is recognized that Mtz metabolism and resistance development in H. pylori is likely to be complex and multifactorial, and that the effects of an inactivated frxA gene could be compensated for by enhanced or decreased expression of other, as-yet-unknown, genes that have similar functions. In transformation experiments, mutated exogenous frxA was inserted into naive strains that may have no such compensatory mechanisms in place and this could result in development of phenotypic Mtz resistance, which possibly would not occur naturally in that strain or in the infected gastric mucosa. It is evident that it is difficult to evaluate the role of a single gene in Mtz resistance, when studied in isolation without considering the complex interplay that may exist between several genes in the artificial environment of the laboratory, and the functions these genes may have in the natural host gastric environment.

A recent study suggested that frxA expression may be negatively regulated by FdxA ferredoxin (Mukhopadhyay et al., 2003). The single adenine deletions in a poly(A) tract frequently observed at nucleotide 53 may indicate an additional regulatory mechanism whereby frxA could be switched on and off. Slipped-strand mispairing is an important means of transcriptional phase variation in a range of H. pylori genes including those involved in lipopolysaccharide synthesis (Appelmelk et al., 1999), the porin gene hopZ (Peck et al., 1999) and filP, a gene encoding the flagellar basal body (Josenhans et al., 2000). As the role of NAD(P)H flavin oxidoreductase in nature remains to be established, so does the significance of this potential switch mechanism.

In conclusion, frameshift mutations in frxA are common both in sensitive and in resistant strains of H. pylori from patients in the UK and are thus unlikely to play a role in the mechanism of Mtz resistance in these cases. Further investigation of frxA expression of the FrxA protein, particularly in relation to other candidate genes in a larger study population, will be essential to further understand their role in the development of the MtzS phenotype.

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REFERENCES


Jeong, J. Y., Mukhopadhyay, A. K., Dailidiene, D. & 20 other authors (2000). Distribution of frxA frameshift mutation 53 in MtzR and MtzS isolates from London (light grey), Wales (hatched) and elsewhere in the UK [dark grey; H. pylori isolates from mid-Essex, Leeds, Portsmouth (England) and Lanarkshire (Scotland)].

Fig. 3. Distribution of frxA frameshift mutation 53 in MtzR and MtzS isolates from London (light grey), Wales (hatched) and elsewhere in the UK [dark grey; H. pylori isolates from mid-Essex, Leeds, Portsmouth (England) and Lanarkshire (Scotland)].


