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

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Mutations in the NAD(P)H flavin oxidoreductase gene (*frxA*) are thought to contribute to the development of metronidazole resistance in *Helicobacter pylori*. To test this further, 44 *frxA* sequences in 18 patient isolate sets of *H. pylori* were examined including a unique collection comprising separated Mtz-sensitive (Mtz^S) and Mtz-resistant (Mtz^R) subpopulations pre-treatment and matched Mtz^R strains post-treatment. Sequences of *frxA* contained frameshift mutations that led to premature protein truncation in at least one strain from most (17/18) patient sets. These mutations were present in all strains, irrespective of Mtz resistotype in 13/18 patients. Frameshift due to a single adenine deletion at nucleotide 53 was the most common mutation and was present in isolates from 11/18 patients. A novel real-time (LightCycler) PCR-based probe hybridization melting-point assay applied to a further 119 isolates confirmed that the frameshift-53 mutation occurred frequently, in 20 % of isolates, and could be present in Mtz^S as well as Mtz^R strains (42 % vs 58 %). This study demonstrates that frameshift mutations occur in Mtz^S strains as well as in Mtz^R strains, and are thus unlikely to cause Mtz resistance.

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).

Abbreviations: Mtz^R, Mtz-resistant; Mtz^S, Mtz-sensitive.

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*

only (Kwon *et al.*, 2001a; Marais *et al.*, 2003). Thus, the exact contribution of *frxA* inactivation to Mtz 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 Mtz 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 *in vitro* Mtz 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 Mtz-containing therapy. Our previous characterization of the Mtz MIC by E-test demonstrated that 7/11 isolates were mixed Mtz^S/Mtz^R before treatment and Mtz^R post-treatment (patients A, B, E, G, H, I, K), 3/11 isolates were Mtz^S before therapy and Mtz^R after (patients C, F, J) and 1/11 was Mtz^S pre-treatment and mixed Mtz^S/Mtz^R post-treatment (patient D). All mixed infections had been purified into separate Mtz^S and Mtz^R 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 Mtz^R and Mtz^S 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 Mtz^S ($n = 61$) or Mtz^R ($n = 58$) (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 °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 FrxF/FrxR (Jeong *et al.*, 2001) and novel primers FrxA2F (5′-AGG TTC GCT CAA ATC ATC A-3′) and FrxA2 R (5′-TTC AAT CAC TTC ATA AAT AAC-3′) were also used to generate *frxA* fragments. Briefly, fragments were amplified in a 100 µl reaction containing 200 ng DNA from culture, 200 µM (each) dNTP (Invitrogen), 0·4 µM each of the appropriate primers (MWG Biotech), 2·0 mM $MgCl_2$, 20 mM Tris/HCl, pH 8·4, 50 mM KCl, 0·2 % (v/v) glycerol, 2 U *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 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s and elongation at 72 °C for 1 min, followed by 5 min at 72 °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 and translated 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 µl reaction containing 20 ng DNA, 1×FastStart DNA Master SYBR Green 1 master mix (Roche Diagnostics), 6 mM $MgCl_2$, 0·5 µM each primer, EFR-1mod (5′-TCT CAA GCG GAA AAA TCC-3′) and frxR(FS) (5′-ATC TTC TTT CAT GCG TTC A-3′) (MWG Biotech), 5 µM 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 °C), by 50 cycles of denaturation (95 °C for 0 s), annealing (45–48 °C for 0 s, temperature increment 4 °C s^{-1}) and extension (72 °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 °C (temperature increment 0·1 °C s^{-1}).

RESULTS AND DISCUSSION

Comparison of translated FrxA amino acid sequences in Mtz^S and Mtz^R strains

The aim of this study was to investigate the potential contribution of naturally occurring *frxA* mutations to Mtz 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 Mtz^R and Mtz^S subpopulations, usually recovered pre-treatment, to establish if either mutations observed post-treatment were also present pre-treatment in the matched Mtz^R subpopulation, or if *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 Mtz^R populations in three patients (C, I and J) and only in the Mtz^S 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

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

frxA mutation (nucleotide position)			FrxA amino acid sequence change (codon)	Patient
Pre-treatment		Post-treatment		
Mtz^S	Mtz^R	Mtz^R		
Frameshift (53)	Frameshift (53)	Frameshift (53)	Stop codon (39)	A, D, E
Frameshift (53)	Frameshift (53)	NA*	Stop codon (39)	G, H
Frameshift (212)	Frameshift (212)	Frameshift (212)	Stop codon (92)	B
Frameshift (211)	Frameshift (211)	Frameshift (211)	Stop codon (91)	K
No mutation	Frameshift (209)	Frameshift (209)	Stop codon (74)	I
No mutation	NA†	Frameshift (209)	Stop codon (74)	C
No mutation	NA†	Frameshift (53)	Stop codon (39)	J
Frameshift (53)	Frameshift (53)	NA‡	Stop codon (21)	L
No mutation	NA†	No mutation	None	F
Frameshift (53)	Frameshift (53)	NA‡	Stop codon (39)	O, P, R
Frameshift (24)	Frameshift (24)	NA‡	Stop codon (39)	M
Frameshift (53)	Frameshift (53)	NA‡	Stop codon (54)	N
Frameshift (24)	No mutation	NA‡	Stop codon (39)	Q

*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 Mtz^R strains were observed pre-treatment.

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 Mtz^S and four Mtz^R 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 Mtz^R strains only (Marais *et al.*, 2003). In contrast, our study demonstrated that such mutations also occur in Mtz^S 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 Mtz^S 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 Mtz^S and Mtz^R 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),

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Patient F  1   MDREQVVALQ HQRFAAKKYD SNRRISQKDW EALVEVGRLA PSSIGLEPWK MLLLKNERMK  60

Patient B  1   MDREQVIALQ HQRFAAKKYD PNRRISQKDW EALVEVGRLA PSSIGLEPWK MLLLKNERMK  60

Patient K  1   MDREQVVALQ HQRFAAKKYD PNRRISQKDW EALVEVGRLA PSSIGLEPWK MLLLKNERMK  60

Patient C  1   MDREQIIALQ HQRFATKKYD PNRRISEKDW EVLVEVGRLA PSSIGLEPWK MLLLKNERMK  60

                                  Δ-A*
Patient N  1   MDREQVVALQ HQRFAAKNTI LIVVFPKRIG KLWLKWGDYP LLQSGLNHGK CFY-         53

                                  Δ-A*
Patient A  1   MDREQVVALQ HQRFAAKNTI LIVVFPKRIG KLWLKWGD-                          38

                                  Δ-AA*
Patient L  1   MDREQVVALQ HQRFAAKIRS -                                             20


Patient F  61  EDLKPMAWGA LFGLEGASHF VIYLARKGVT YDSDYVKKVM HEVKKRDYDT NSRFAQIIKN  120

                            Δ-T*
Patient B  61  EDLKPMAWGA LFWFGGSEPF CHLSCAKRRY L-                                 91

                           Δ-CT*
Patient K  61  EDLKPMAWGA FWFRGSEPFC HLSCAKRRYL -                                   90

                        Δ-G*
Patient C  61  EDLKPMAWGG FLV-                                                     73


Patient F  121 FQENDMKLNS ERSLFDWASK QTYIQMANMM MAAAMLGIDS CPIEGYDQEK VEAYLEEKGY  180


Patient F  181 LNTAEFGVSV MACFGYRNQE ITPKTRWKTE VIYEVIE- 217
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Fig. 1. Examples of *H. pylori* FrxA amino acid sequences determined for each frameshift mutation type observed in different patient sets. *Nucleic acid sequence changes that caused amino acid alterations.

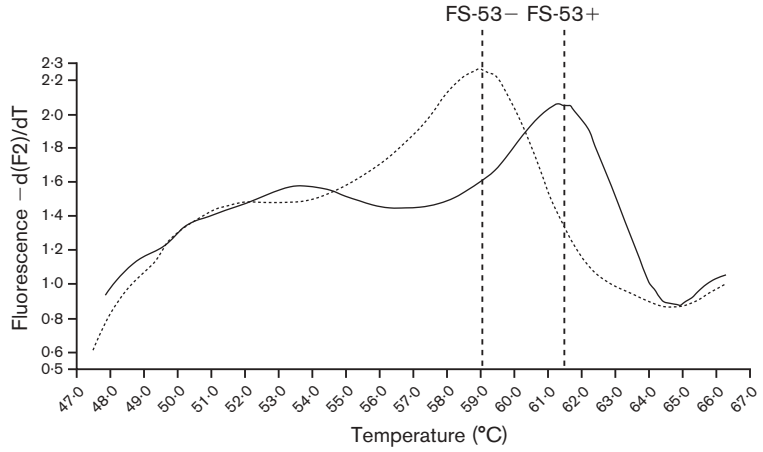

Fig. 2. Melting peaks generated by probe hybridization melting-point analysis (FS-53 LightCycler assay) to screen for frameshift mutations at nucleotide 53 in *H. pylori frxA*. FS-53+ indicates isolates containing the frameshift mutation and FS-53− indicates isolates lacking the adenine deletion at position 53.

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 Mtz^S 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 53 was at least 20·2 % in UK isolates. Furthermore, of the 24 strains containing FS-53, 14/24 (58·3 %) were Mtz^R but 10/24 (41·7 %) were Mtz^S (Fig. 3), providing further evidence that FrxA inactivation alone is unlikely to cause Mtz resistance.

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

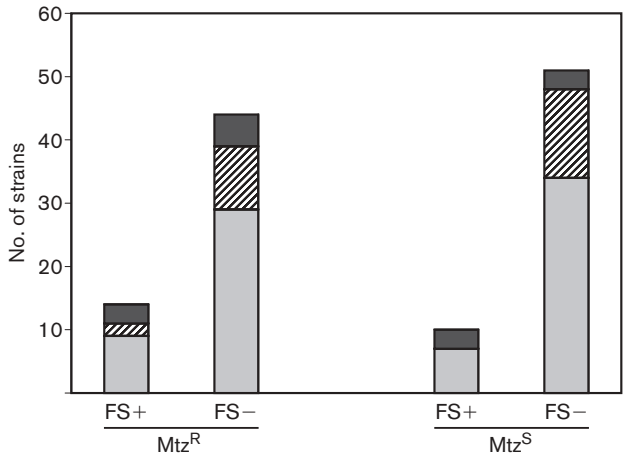

Fig. 3. Distribution of *frxA* frameshift mutation 53 in Mtz^R and Mtz^S isolates from London (light grey), Wales (hatched) and elsewhere in the UK [dark grey; *H. pylori* isolates from mid-Essex, Leeds, Portsmouth (England) and Lanarkshire (Scotland)].

study that demonstrated inactivated *frxA* genes did not always transform *H. pylori* phenotype from Mtz^S to Mtz^R (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 extrogenous *frxA* was inserted into naïve 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 *fliP*, 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* and 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 Mtz^R phenotype.

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