Proton motive force-dependent efflux of tetracycline in clinical isolates of *Helicobacter pylori*

Mona Anoushiravani,¹ Tahereh Falsafi¹ and Vahid Niknam²

Correspondence

Tahereh Falsafi

tfalsafi@yahoo.com

¹Department of Biology, Alzahra University, Tehran, Iran

²Department of Biology, Tehran University, Tehran, Iran

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The aim of this study was to evaluate the role of proton motive force (PMF)-dependent efflux in resistance of *Helicobacter pylori* to tetracycline (Tet). Tet MIC was determined by agar dilution in the presence and absence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an inhibitor of PMF. Antibiotic accumulation was conducted in the presence or absence of CCCP and the fluorescence of the accumulated antibiotic was measured by spectrofluorometry. In the presence of CCCP, antibiotic accumulation was increased by 2–17-fold in 17/20 Tet^r isolates and by 3–10-fold in four of five high-level-resistant mutants. Correlation was observed between this increase and diminution of MIC with CCCP. PMF-dependent efflux mechanisms therefore appear to play an important role in the resistance of clinical isolates of *H. pylori* to Tet.

INTRODUCTION

The antibiotics commonly used to eradicate *Helicobacter pylori* infection include amoxicillin (Amx), clarithromycin (Cla), tetracycline (Tet) and metronidazole (Mtz), plus a proton pump inhibitor (Graham & Qureshi, 2000; Loffeld & Fijen, 2003). As Tet can cause staining of the permanent teeth and interfere with bone growth, it is not recommended for young children. However, Tet^r *H. pylori* strains have increasingly been isolated in both adults and children, especially in developing and Asian countries (Kwon *et al.*, 2000; Mendonca *et al.*, 2000; Wu *et al.*, 2000, 2005).

Tet is an inhibitor of protein synthesis with a broad spectrum of activity against both Gram-negative and Gram-positive bacteria. It binds to the 30S ribosomal subunit and blocks the binding of aminoacyl-tRNA, preventing the synthesis of nascent peptide chains (Chopra & Roberts, 2001). The mechanisms of bacterial resistance to Tet have been widely studied in many bacteria. Four different Tet resistance mechanisms have been described: (i) a defect in the uptake of the antibiotic; (ii) an increase in its efflux; (iii) decreased antibiotic binding, either by changes in ribosomal protection proteins or by mutations in the 16S rRNA tetracycline-binding site; and (iv) enzymic inactivation of Tet (Chopra & Roberts, 2001; Nikaido, 1998). In *H. pylori*, involvement of the first three mechanisms in resistance to Tet has been proposed; however, the best-studied mechanism remains mutation in the 16S rRNA gene, which is based on a single, double or triple base-pair substitution in adjacent 16S rRNA gene

residues (Dailidiene *et al.*, 2002; Glocker *et al.*, 2005; Kwon *et al.*, 2000; Lawson *et al.*, 2005; Trieber & Taylor, 2002). These mutations are located in the primary binding site of tetracycline, and probably affect the affinity of the drug for the ribosome, thus reducing its inhibitory effect (Lawson *et al.*, 2005; Nonaka *et al.*, 2005).

In many Gram-negative bacteria, resistance to tetracycline is also related to an energy-dependent efflux of Tet across the cell membrane (Chopra & Roberts, 2001; Nikaido, 1998). Although several investigators have studied the probable role of active efflux in the intrinsic resistance of *H. pylori* to antibiotics (Bina *et al.*, 2000; Kutschke & de Jonge, 2005; Li & Dannelly, 2006; van Amsterdam *et al.*, 2005), there are no data concerning the precise role of this mechanism in resistance to Tet. In a recent study, Wu *et al.* (2005) examined whether Tet resistance was associated with an energy-coupled active efflux mechanism. In this work, they were not able to observe the role of active efflux in resistance to Tet, but they proposed alteration of membrane permeability as a possible mechanism responsible for resistance of *H. pylori* to Tet.

The aim of our study was to evaluate the role of proton motive force (PMF)-dependent efflux in the primary resistance of *H. pylori* to Tet in clinical isolates obtained from children.

METHODS

Bacterial strains and culture conditions. *H. pylori* isolates were obtained by screening of 112 *H. pylori* strains isolated during 1997–2008 from 250 children at the Children Medical Center of Tehran. Bacteria were routinely grown on modified Campy blood agar and Belo Horizonte agar (Merck) as described previously (Falsafi *et al.*,

Abbreviations: Amx, amoxicillin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Cla, clarithromycin; Mtz, metronidazole; PMF, proton motive force; Tet, tetracycline.

2007). Colonies were identified by Gram staining, biochemical tests and PCR using *H. pylori*-specific primers *16sRNA* (Falsafi *et al.*, 2009) and *ureC* (Cinnagen), as described previously (Falsafi *et al.*, 2009).

MIC determination. Susceptibility to Tet (Sigma) was determined by establishing the MIC value according to the recommendations of the Clinical and Laboratory Standards Institute (Best *et al.*, 2003). Plates were prepared with Mueller–Hinton (MH) agar (Merck) plus 7% fresh sheep blood and Tet at a concentration of 0.5–128 mg l⁻¹. *H. pylori* isolates were grown for 3 days on modified Campy blood agar plates, harvested and suspended in sterile saline to obtain a McFarland 2 standard ($\sim 1 \times 10^8$ – 2×10^8 c.f.u. ml⁻¹). The MH agar plates were spread with bacterial suspension and incubated for 2–3 days at 37 °C under microaerobic conditions. Antibiotic-free plates were inoculated during each series of tests to confirm the validity of the inoculum and to observe the presence of any contamination. *H. pylori* ATCC 26695 was used as the quality-control organism and measurements were repeated three times to confirm the results. The resistance cut-off point for Tet was an MIC of ≥ 4 mg l⁻¹ (King, 2001; Lawson *et al.*, 2005).

Determination of MIC was also performed in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Sigma), an inhibitor that disrupts the proton gradient across the membrane. For this purpose, inoculated plates were dried for 5 min and discs containing 30 µl CCCP at a concentration of 100 µM were placed on the agar surface. A control plate containing 100 µM CCCP showed that this concentration of CCCP did not produce any collapse of bacterial growth. The plates were incubated for 2 days, after which the MIC was defined as the lowest concentration of antibiotic that showed growth inhibition around the CCCP disc.

Determination of cross-resistance to Mtz, Amx and Cla by the disc diffusion method. All Tet^R *H. pylori* isolates were also tested by the disc diffusion method for susceptibility to Tet, Mtz, Cla and Amx. Discs containing Tet (30 µg), Amx (30 µg), Mtz (5 µg) and Cla (15 µg) were purchased from Himedia. Five microlitres of bacterial suspension at a concentration of a McFarland 2 standard was spread on sheep blood MH agar plates, the discs were added and the diameters of the zones of inhibition were measured after 3 days. Quality control was ensured by using the following organisms: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *H. pylori* ATCC 26695.

Inhibition zones of ≥ 30 , ≥ 14 , ≥ 10 and ≥ 14 mm were interpreted as *H. pylori* resistance to Tet, Mtz, Cla and Amx, respectively (Lawson *et al.*, 2005; McNulty *et al.*, 2002).

Accumulation assay. Accumulation of Tet in *H. pylori* was determined as described previously (Lin *et al.*, 2002) with some modifications. *H. pylori* strains were grown on Campy blood or MH agar plates for 2 days, harvested and resuspended in 50 mM sodium phosphate buffer (pH 7.2) with a density equivalent to a McFarland 4 standard (OD₆₀₀=0.6) corresponding to 1.2×10^9 c.f.u. ml⁻¹. The cell suspension was incubated for 10 min at 37 °C and antibiotic-uptake assays were initiated by the addition of Tet to 500 µl bacterial cell suspension at a final concentration of 20 µg ml⁻¹. At 10 min after antibiotic addition, CCCP was added to half of the reaction mixture at a final concentration of 200 µM and the other half was used as a control (no CCCP). After 20 min, each of the collected samples was diluted in 1.5 ml ice-cold sodium phosphate buffer and centrifuged for 15 min at 6000 g at 4 °C. The supernatants were discarded and the pellets were resuspended in 2 ml 0.1 M glycine hydrochloride (pH 3.0) and shaken at 25 °C for 16 h to extract the accumulated antibiotic. After centrifugation at 6000 g for 15 min, the supernatant was used to measure the fluorescence of Tet with a Shimadzu RF 5000 spectrofluorometer (Shimadzu Scientific Instruments) at excitation and emission wavelengths of 400 and 450 nm, respectively. The

concentration of Tet in the supernatant was measured by comparison with a standard curve of Tet in 0.1 M glycine hydrochloride. The results were expressed as ng Tet (mg dry weight bacteria)⁻¹.

Selection of *H. pylori* high-level-resistant mutants. Five clinical *H. pylori* isolates that were weakly or moderately resistant to Tet were grown on Campy blood agar plates and consecutively subcultured on plates supplemented with increasing concentrations of Tet. After growth, one colony of each isolate that was capable of growth at the highest concentration of Tet was picked and tested for a stable high MIC. These selected mutants were subjected to the studies described above for clinical isolates.

RESULTS AND DISCUSSION

A total of 112 *H. pylori* isolates were screened and 20 Tet^R isolates with MICs of 4–64 mg l⁻¹ were isolated. Two isolates susceptible to Tet, Mtz, Amx and Cla were used as negative controls (1B and 17B). Tet^R isolates were classified as low-, intermediate- or high-level Tet^R according to their MICs. Among them, 14 (70%) showed cross-resistance to Mtz, but no resistance was observed for Amx and Cla (Table 1). In the presence of CCCP, the Tet MIC was decreased by one to two dilutions for 17/20 isolates (Table 2). These 17 Tet^R isolates with a CCCP effect on MIC showed PMF-dependent efflux of Tet manifested by a

Table 1. Tet MICs in relation to cross-resistance to Amx, Mtz and Cla

Resistance to Tet was determined by the agar dilution and disc diffusion methods. S, Sensitive; R, resistant; I, intermediate.

Strain no.	Amx	Mtz	Cla
Low-level resistance (MIC 4–8 µg ml ⁻¹)			
12M	S	S	S
15M	S	I	S
27M	S	S	S
10II	S	I	S
18B	S	R	S
58II	S	R	S
23M	S	S	S
Moderate resistance (MIC 16 µg ml ⁻¹)			
19M	S	S	S
20M	S	R	I
24M	S	R	S
25M	S	R	I
26M	S	R	S
28M	S	R	S
21D	S	R	S
2N	S	R	I
High-level resistance (MIC 32–64 µg ml ⁻¹)			
17M	S	R	S
18M	S	R	S
21M	I	R	S
1N	S	R	I
3N	I	R	S

Table 2. Tet MICs in relation to accumulation results in the presence and absence of CCCP

Strain no.	MIC		Accumulation*		Increase†
	−CCCP	+CCCP	−CCCP	+CCCP	
Low-level resistance					
12M	8	4	0.08	1.33	16.6-fold
15M	8	4	0.69	1.14	1.5-fold
27M	8	4	0.90	2.70	3-fold
10II	8	8	0.58	0.66	No change
18B	4	2	0.50	4.39	8.8-fold
58II	4	4	0.48	0.52	No change
23M	4	2	0.38	0.83	2-fold
Moderate resistance					
19M	16	8	1.46	3.45	2.4-fold
20M	16	8	0.14	0.29	2-fold
24M	16	8	0.63	1.9	3-fold
25M	16	8	0.26	0.55	2-fold
26M	16	4	0.32	1.2	~4-fold
28M	16	4	1.04	4.48	4-fold
21D	16	8	0.22	0.45	2-fold
2N	16	16	1.64	1.84	No change
High-level resistance					
17M	32	8	0.33	2.27	7-fold
18M	32	8	0.32	0.67	2-fold
21M	32	8	0.14	0.27	2-fold
1N	32	8	0.39	2.5	6-fold
3N	64	16	0.65	2.34	~4-fold
Susceptible					
1B	0.5	ND	0.05	0.055	No change
17B	1	ND	0.10	0.11	No change
26695	2	ND	0.15	0.15	No change

ND, Not determined.

*Measured as ng Tet (mg dry weight bacteria)^{−1}.

†Fold increase in concentration of accumulated Tet in the presence of CCCP.

2–17-fold increase in the amount of Tet (mg dry weight bacteria)^{−1} in the presence of CCCP (Table 2). No significant difference was observed between low-, intermediate- and high-level Tet^R isolates regarding the amount of energy-dependent efflux of Tet. High-level Tet^R mutants that were selected on the basis of growth on increasing concentrations of Tet and that had stable MICs showed an MIC that was increased by one to three dilutions. Their cross-resistance profiles remained unchanged, except for mutant 23M, which acquired resistance to Mtz during passage (Table 3). In the presence of CCCP, the MIC for Tet was decreased by two dilutions for all of the high-level Tet^R mutants, except for the 23M mutant, which remained unchanged. In the presence of CCCP, the concentration of accumulated Tet in the mutants increased 3–10-fold compared with their parental strains, except for 23M, which showed a decrease (Table 3).

Although Tet resistance is rarer in *H. pylori* than in other bacteria, its emergence varies according to geographical area. For example, in Western countries, Tet resistance is

rare (Osato *et al.*, 2001), but in Japan and Korea, 5–7 % resistance has been observed, and in China high levels of resistance (59 %) have been found (Wu *et al.*, 2000). We screened 112 isolates and found 18 % resistance to Tet with MICs ranging from 4 to 64 mg l^{−1}. Seventy per cent of these isolates were cross-resistant to Mtz, similar to results observed in Korea (Kim *et al.*, 2001). Although Trieber & Taylor (2002) have proposed that the known Tet^R loci are not genetically linked to the Mtz^R locus, it is difficult to extrapolate this suggestion to all cases, as one mutant (23M mutant) that was co-resistant to both antibiotics was selected in this work.

In the present work, a significant increase in the concentration of accumulated Tet in the presence of CCCP was observed for 17 Tet^R isolates, suggesting that efflux pumps energized by PMF were functional in these isolates. However, no significant difference was observed between low-, intermediate- and high-level Tet^R isolates regarding the amount of active efflux (Table 2). This suggests that the activity of efflux pumps may not be

Table 3. Tet MICs, cross-resistance and accumulation of five high-level Tet^R mutants compared with the parental isolates

Strain no.	Tet MIC		Cross-resistance to Amx–Mtz–Cla	Accumulation*		Increase†
	–CCCP	+CCCP		–CCCP	+CCCP	
18M	32	8	S R S	0.32	0.67	2-fold
18M-mut	64	16	S R S	0.37	1.97	5.3-fold
20M	16	32	S R I	0.14	0.29	2-fold
20M-mut	64	32	S R I	0.29	1.5	5-fold
21M	32	8	I R S	0.14	0.27	2-fold
21M-mut	64	16	S R I	0.75	2.6	3.5-fold
23M	4	2	S S S	0.38	0.83	2-fold
23M-mut	32	32	S R S	0.16	0.32	2-fold‡
25M	16	8	S R I	0.26	0.55	2-fold
25M-mut	32	8	S R S	0.35	2.4	7-fold

*Measured as ng Tet (mg dry weight bacteria)^{−1}.

†Fold increase in concentration of accumulated Tet in the presence of CCCP.

‡A decrease of ~2.5-fold (in the presence of CCCP) compared with the 23M parent strain.

related to the MIC and that more than one mechanism may be responsible for the development of high-level Tet resistance. The increase in amount of proton-dependent efflux in four of the five high-level-resistant mutants (Table 3) may indicate that, during passage, the effect of pressure of the antibiotic selected the strains with the more active efflux pumps. However, during step-wise growth on Tet, the MIC of strain 23M was increased from 4 to 32, although its efflux activity was widely decreased (Table 3), which may relate to alteration of its permeability. The phenotype observed in the case of this mutant is similar to those observed by Wu *et al.* (2005), suggesting that decreased accumulation of Tet may be one of the Tet resistance mechanisms. However, the key finding of this work is that CCCP, an inhibitor of PMF, increased Tet accumulation in clinical isolates and in high-level-resistant mutants. This is different from the findings of Wu *et al.* (2005): whilst they showed decreased accumulation in resistant strains, they were not able to demonstrate the PMF-dependent efflux of Tet.

Thus, the results of the present study provide data in favour of active efflux as a significant mechanism in the resistance of clinical isolates of *H. pylori* to Tet.

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