Infection and Drug Resistance

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ORIGINAL RESEARCH

A Newly Discovered Drug Resistance Gene *rfaF* In *Helicobacter pylori*

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Background: The purpose of this study was to understand the function of *rfaF* gene in *Helicobacter pylori* antibiotic resistance.

Methods: The gene homologous recombination method was used for knockout and complementation of *H. pylori rfaF* gene. Various constructed strains were analysed for drug sensitivity to amoxicillin (AMO), tetracycline (TET), clarithromycin (CLA), metronidazole (MET), levofloxacin (LEV), and chloramphenicol (CHL) by agar plate dilution method. Drug sensitivity was further confirmed using a growth inhibition curve. Ethidium bromide (EB) accumulation experiments were performed to assess cell membrane permeability. PCR and sequence analysis were used to detect the *rfaF* gene.

Results: The minimum inhibitory concentrations (MIC) of TET, CHL, AMO, and CLA in 11,637 *rfaF* knockout strain (Δ*rfaF* strain) were 4, 4, 2, and 2 times higher than those in 11,637 wild type (WT) strain, respectively. A multidrug-resistant (MDR) Δ*rfaF* strain also displayed the same trend; however, the degrees of increase were relatively small. Growth inhibition experiments indicated that the growth of the 11,637 Δ*rfaF* strain was higher with antibiotics at the MIC of the 11,637 WT strain than that of 11,637 *rfaF*-complemented strain (Δ*rfaF*/*rfaF* strain), whereas the 11,637 WT strain did not exhibit any growth. The 11,637 Δ*rfaF* strain was significantly reduced compared with the cumulative EB fluorescence intensity of the 11,637 WT and of 11,637Δ*rfaF*/*rfaF* strain, and the same trend appeared in the MDR strain. Among the 10 clinical strains, 9 clinical strains were found to have mutations in the conserved sequence of *rfaF* amino acids.

Conclusion: We found a new drug resistance gene, *rfaF*, in *H. pylori*, which changes the permeability of cell membrane to confer cross-resistance to AMO, TET, CLA, and CHL and is involved in clinical strain drug resistance. It can be used as a drug target.

Keywords: *rfaF*, amoxicillin, clarithromycin, tetracycline, resistance

Introduction

Helicobacter pylori (*H. pylori*), which is a spiral, gram-negative bacterium, has affected more than half of the world population. It is known to be involved in the pathogenesis of chronic gastritis, peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma.[1,2] Clinically, two or three antibiotics out of the following list: amoxicillin (AMO), tetracycline (TET), clarithromycin (CLA), metronidazole (MET), and levofloxacin (LEV) combined with a proton-pump inhibitor (PPI) or bismuth salts are typically used to kill *H. pylori*.[3,4] With the widespread use of a large number of antibiotics, *H. pylori* has displayed high antibiotic resistance rate in recent years.[5,6]

In addition to drug target-binding point mutations and effective efflux, an important mechanism of *H. pylori* antibiotic resistance is to reduce drug permeability.[7–10] The

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rfaF (*waaF*) gene, mutated to produce a bacterium with a deep coarse lipopolysaccharide (LPS) phenotype, was identified to function as a core heptose transferase.[11] Previous research has demonstrated that the LPS core biosynthetic pathway gene *rfaF* is involved in drug resistance.[12–14] Structural analysis identified the activity of HP1191 in *H. pylori* 26,695 genome as a heptosyltransferase and a *rfaF* homolog. The *rfaF* was confirmed to be involved in biosynthesis of the inner-core region of *H. pylori* LPS.[15–17] However, the role of *rfaF* in drug tolerance has been unclear in *H. pylori*. Therefore, the purpose of this study was to investigate the mechanism by which *rfaF* function affects drug sensitivity.

Methods

Bacterial Culture

H. pylori 11,637 and 10 clinical isolates of *H. pylori*, which were kindly offered by the Department of Medical Microbiology of Fujian Medical University, were used in this study. The strains were conserved at −80°C at 20% sucrose and 50% fetal bovine serum (FBS). The strains were cultured under a microaerobic environment (5% O_2, 10% CO_2, 85% N_2) at 37°C on Campylobacter agar plates containing 7% sheep blood. The liquid culture medium for *H. pylori* consisted of Brucella broth and 10% FBS, and the cells were incubated in a shaker at 120 rpm at 37°C. The mutant strains were cultured on agar plates with kanamycin (MP Biomedicals, LLC) at 5 μg/mL or CHL (Inalco S.p.A., Milano Italy) at 8 μg/mL.

Construction Of *rfaF* Mutant And Complemented Strains By Allelic Exchange Mutagenesis

To construct a *rfaF* knockout mutant of *H. pylori* 11,637, an upstream 718 bp fragment and a downstream 777 bp fragment of *rfaF* were amplified from wild type (WT) 11,637 genomic DNA with primer F1, R1, F2, and R2, respectively. These two fragments and a kanamycin resistance cassette, which was amplified with F3 and R3, were ligated into a pBluescript II vector by ligase independent cloning (LIC) with ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd). The vectors were subsequently introduced into *H. pylori* 11,637 strains by electrotransformation. Chromosomal DNA of the transformants was checked by PCR with primers F4 and R4. The primers used in this study are listed in Table 1.

To construct an *rfaF* partial knockout mutant of a *H. pylori* multidrug-resistant (MDR) strain, which was randomly selected from 10 clinical isolates and numbered as FZ068, the same upstream fragment and a 537 bp downstream fragment from +754 to +1263 of *rfaF* ORF were amplified from WT MDR genomic DNA with primer F1, R1, F5, and R5, respectively. These two fragments and a kanamycin resistance cassette, which was amplified with F6 and R6, were ligated into a pBluescript II vector by LIC with ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd). The vectors were subsequently introduced into *H. pylori* MDR strains by electrotransformation. Chromosomal DNA of the transformants was checked by PCR with primers F7 and R7 (Table 1).

To construct a *H. pylori* 11,637 *rfaF*-complemented strain, an upstream 534 bp fragment, a 1228 bp fragment of *rfaF* (containing *rfaF* 150 bp promoter region and the whole *rfaF* ORF), and a downstream 813 bp fragment were amplified from wild type 11,637 genomic DNA with primer F8, R8, F9, R9, F10, and R10, respectively. These three fragments and a chloramphenicol resistance cassette which was amplified with F11 and R11 were ligated into a pBluescript II vector by LIC with ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co., Ltd). The vectors were then introduced into 11,637 *rfaF* knockout strains by electrotransformation. Chromosomal DNA of the transformants was checked by PCR with primers F12 and R12 (Table 1).

Antibiotic Susceptibility Testing

The MICs of AMO, TET, CLA, MET, LEV, and CHL for all strains were determined by the agar dilution method reported by Osato, et al.[18] Briefly, 2 μL of the 2.0 McF suspension cultures was inoculated on a Mueller-Hinton agar plate containing twofold dilutions of an antibiotic. All the plates were incubated at 37°C under microaerobic conditions, and the MIC values were determined after 72 hrs. *H. pylori* 11,637 was used as a reference strain for quality control of antibiotic susceptibility testing. Each experiment was repeated at least three times.

Determination Of Growth Curves And Growth Inhibition Curves

The growth curve was determined as described in previous literature.[19] To monitor the growth curve of bacteria, the initial optical density (OD_{600}) value of the bacterial suspension in Brucella broth was measured as 0.1 and then the bacteria were shake cultured at 37°C for 96 hrs. The

Table 1 PCR Primers Used For Construction In This Study

Primers	Sequence(5′–3′)	Size Of Product (bp)	Positions In 26,695 DNA Sequence(No. NC_000915.1)
F1	CTATAGGGCGAATTGGGTACCCCCATTCAAGCCAATTTTTTCC	718	From 1,261,390 to 1,262,065
R1	TAAAACCGCCCAGTCTCGAG ACGCATGCGTTTGGGTGC		
F2	TCTCGAGGCCGCTCACACTCTTTTAGAAGA	777	From 1,263,056 to 1,263,804
R2	AAAGCTGGAGCTCCACCGCGGTTTTAAAGGGCCTAAATGACGC		
F3	ATGCGTCTCGAGACTGGGCGGTTTTATGGACA	1214	
R3	CTAAAAGAGTGTGAGCGGCCTCGAGAAATTAAAAATGAAGTTTTAGCACG TG		
F4	AAGGGCTATTGATGAGCGTA	1066 or 1199	From 1,262,025 to 1,263,090
R4	GGCGTTTAAGGCTCTTCT		
F5	TCTCGAGTGCGGGAAAACAAGCATTG	537	From 1,262,771 to 1,263,279
R5	AAAGCTGGAGCTCCACCGCGGACTCAGTGGCCTAATGGCTTTGACT		
F6	ATGCGTCTCGAGACTGGGCGGTTTTATGGACA	1214	
R6	CAATGCTTGTTTTCCCGCACTCGAGAAATTAAAAATGAAGTTTTAGCACG TG		
F7	AAGGGCTATTGATGAGCGTA	1068 or 2257	From 1,262,025 to 1,263,092
R7	AAGGCGTTTAAGGCTCTTC		
F8	CTATAGGGCGAATTGGGTACCTTGAGTGTTGAGAATTTAGAAAAATGG	534	From 174,691 to 175,187
R8	GTTTCAGTCGCTCGAGTCACTCTGTGTTTTCTCGCTCCA		
F9	CACAGAGTGACTCGAGCGACTGAAACTTTCGCAAGCA	1228	From 1,261,884 to 1,263,085
R9	GTGCCGATCATTAAGGCTCTTCTAAAAGAGTGTGAGC		
F10	GACGATATGATCATCTCGAGCCATGAAAACAAAGCCTTAAATTTC	813	From 173,857 to 174,631
R10	AAAGCTGGAGCTCCACCGCGGCGCAAACGGCCAATGATC		
F11	AGAGCCTTAATGATCGGCACGTAAGAGGTTC	1050	
R11	TCATGGCTCGAGATGATCATATCGTCAATTATTACCTCCA		
F12	TGGAATCTTTGGAGCGAG	594 or 2852	From 174,112 to 174,705
R12	AGCGATTGGATGCGAGCTA		
F13	CTCAACACTTTAGGGGTAATCAT	1347	From 1,261,953 to 1,263,299
R13	AAACCTAAAAACTCAGTGGTCTAA		

Note: Underlined part indicates overlapping DNA sequences.

OD_{600} value of the bacterial suspension was recorded every 8 h. Each experiment was repeated at least three times.

To analyse the growth inhibition curve, the *H. pylori* strains were inoculated at initial OD_{600} value of 0.1 in Brucella broth, which contained different antibiotics, and then the bacteria were further cultured for 96 hrs at 37°C with shaking, and the OD_{600} value of the bacterial suspension was recorded every 8 hrs. Each experiment was repeated at least three times.

Determination Of The Accumulation Of Ethidium Bromide (EB)

The EB assay was modified as described previously.[20] *H. pylori* was cultured on Campylobacter blood agar plates for 48 hrs. The cells were subsequently washed twice with PBS (pH 7.0). The cells were resuspended in 1 mL of PBS (pH 7.0) to an OD_{600} of 0.4 and were incubated at 37°C for 30 mins in the presence of 10 μM carbonyl cyanide m-chlorophenylhydrazine (CCCP) to deplete cells of metabolic energy. Subsequently, the cells were washed three times with PBS (pH 7.0) and 10 μg/mL of EB was added. At 30 time points in 30 mins, EB fluorescence intensity was measured by using Synergy™ HT (BioTek®) with an excitation measurement wavelength at 530/25 nm and an emission wavelength at 590/35 nm.

Sequence Data Analysis

The *rfaF* HS PCR products amplified from DNA of 11,637 and 10 clinical strains with primers F13 and R13 were ligated into the PLB plasmids. The ligation plasmids were sent to SunyBiotech Co., Ltd (Fuzhou) for sequence analysis. Finding conserved domains in 26,695 *rfaF* amino acid sequence (NCBI Reference Sequence: NP_207982.1) was performed by NCBI Conserved Domain Search (CD-Search). We used CLC Sequence Viewer 7 software for amino acid conserved domain comparison analysis.

Statistical Analysis

Data are presented as mean ± standard errors of mean. Unpaired *t*-test was used for statistical analysis of the two groups. $P < 0.05$ was considered statistically significant. GraphPad Prism software 6.07 was used to analyse the results.

Ethical Approval

This study was approved by the Fujian Medical University Biomedical Research Ethics Committee ([2012] Fujian Medical University Ethics Review No. (52)).

Results

rfaF Conferred Cross-Resistance To AMO, TET, CLA, And CHL

Assessment Of Susceptibility To Antimicrobial Agents In Various Types Of Strains

In order to assess the drug tolerance of *rfaF*, we constructed knockout and complemented strains of *rfaF*. The MIC level was determined by agar plate dilution method. Using CHL as a screening marker for the construction of *rfaF*-complemented strain, it was unexpectedly found that 11,637 Δ*rfaF* was resistant to CHL; therefore, we increased the amount of CHL to 8 μg/mL. It was identified that in 11,637 and MDR strains, MIC levels of Δ*rfaF* strain did not change compared with those of MEM and LEV of WT strain, but interestingly, MIC levels of Δ*rfaF* strain increased to varying degrees compared with those of AMO, TET, CLA, and CHL of WT strain (Table 2). The MICs of TET, CHL, AMO, and CLA of 11,637 Δ*rfaF* strain were 4, 4, 2, and 2 times higher than those of 11,637 WT strain, respectively. By successfully constructing the 11,637 Δ*rfaF*-complemented strain, MIC values of AMO, TET, CLA, and CHL in 11,637 Δ*rfaF*/*rfaF* strain decreased compared with those in 11,637 Δ*rfaF*. These results imply that *rfaF* confers cross-resistance to AMO, TET, CLA, and CHL but not to MEM and LEV, pointing out a unique resistance mechanism.

Growth Inhibition Experiment

These drug susceptibility results were further confirmed by growth inhibition experiments. Growth curve showed that 11,637 Δ*rfaF* strain grew faster than 11,637 WT and 11,637 Δ*rfaF*/*rfaF* strain (Figure 1A). We treated the 11,637 WT, 11,637 Δ*rfaF*, and 11,637 Δ*rfaF*/*rfaF* strain with three antibiotics (AMO, TET, and CLA) at the MIC of the 11,637 WT strain and then observed their growth inhibition. We found that compared with the growth curve of the 11,637 Δ*rfaF* strain, 11,637 WT was significantly inhibited, and 11,637 Δ*rfaF*/*rfaF* strain was slightly inhibited (Figure 1B–D).

Overall, these results confirm that *rfaF* mutation leads to more resistance to AMO, TET, and CLA.

rfaF Changed Cell Membrane Permeability

rfaF is involved in lipopolysaccharide (LPS) synthesis of *H. pylori*. In addition, changes in LPS structure cause changes in cell membrane permeability leading to changes in drug concentration in the cell membrane, which suggests that *H. pylori rfaF* may alter cell membrane permeability to drugs. Therefore, in order to figure out the resistance mechanism of *rfaF*, we compared the cumulative fluorescence intensity of EB on the cell membranes of 11,637 WT, 11,637 Δ*rfaF*, 11,637 Δ*rfaF*/*rfaF*, MDR WT, and MDR Δ*rfaF* strains. We found that compared with WT strain, 11,637 Δ*rfaF* and MDR Δ*rfaF* strains showed reduced EB cumulative fluorescence intensity. The change in degree of EB fluorescence cumulative intensity between

Table 2 MIC Values Of Various Types Of *H. Pylori* To Antimicrobial Agents

Antibiotic	11,637			MDR(NO.FZ068)	
	WT	**Δ*rfaF***	**Δ*rfaF*/*rfaF***	**WT**	**Δ*rfaF***
AMO	0.064	0.128	0.0.096	0.125	0.1875
TET	0.125	0.5	0.25	0.25	0.375
CLA	0.125	0.25	0.1875	128	192
MEM	1	1	1	256	256
LEV	0.5	0.5	0.5	8	8
CHL	1	4	2	2	3

Note: Drug concentration unit is (μg/mL).
Abbreviations: WT, wild strain; Δ*rfaF*, *rfaF* knockout strain; Δ*rfaF*/*rfaF*, *rfaF* -complemented Strain; AMO, amoxicillin; TET, tetracycline; CLA, clarithromycin; MEM, metronidazole; LEV, levofloxacin; CHL, chloramphenicol.

11,637 WT strain and 11,637 Δ*rfaF* strain is greater than that between MDR WT strain and MDR Δ*rfaF* strain, which was consistent with drug sensitivity change trends (Figure 2). Compared with 11,637 Δ*rfaF* strain, EB cumulative fluorescence intensity of 11,637 Δ*rfaF*/*rfaF* strain was significantly increased. These results indicate that *rfaF* knockout resulted in a decrease in EB permeability through the cell membrane. Therefore, we conclude that *rfaF* knockout reduces drug sensitivity because its mutation decreases the permeability of the cell membrane to the drug, which results in a decrease in drug concentration in the cell membrane.

High Mutation Rate Of Amino Acid Conserved Domains Of *rfaF* From Clinical Isolates Associated With Antibiotic Resistance

To determine whether the target gene *rfaF* mutation is also involved in the resistance of clinical isolates, we compared the amino acid sequence of the target protein of the clinical strains with the conserved amino acid sequence of the target protein in NCBI. Insertions, deletions, and amino acid substitutions found only in highly conserved portions of the target protein in clinical isolates are believed to impart a change in

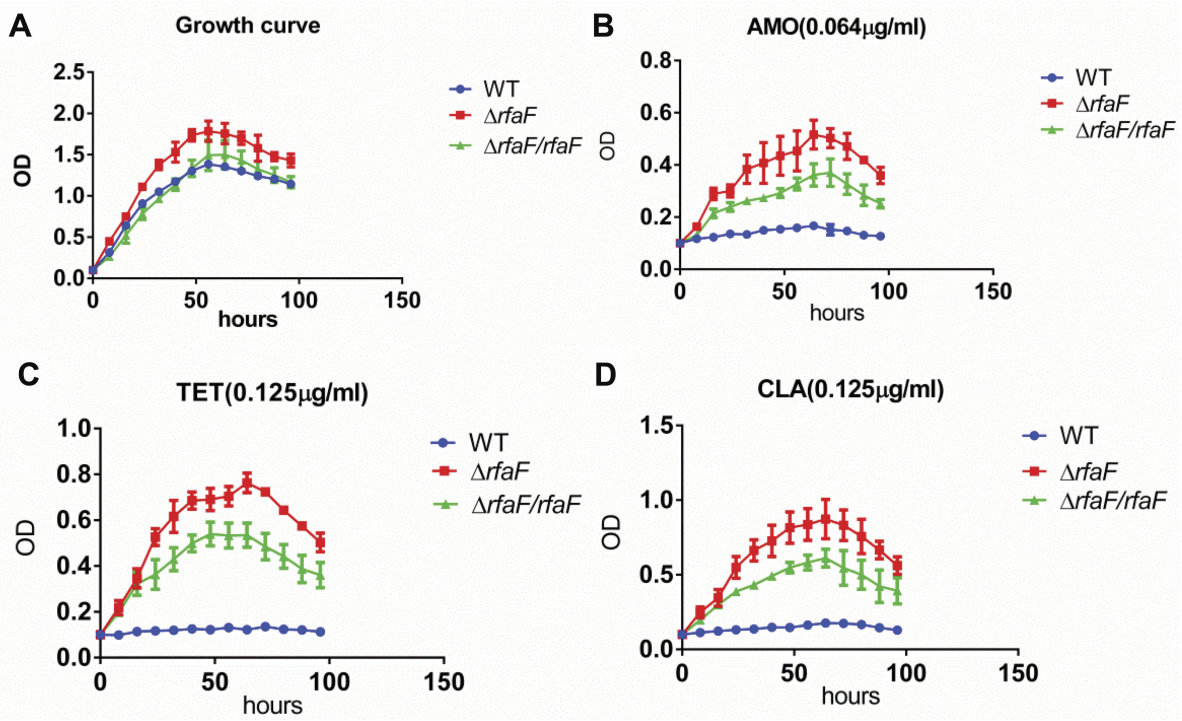

Figure 1 Growth curve of various types of *H. pylori* 11,637 with no drug or with drug.
Notes: (**A**) Growth curve without drug or with (**B**) AMO (0.064 μg/mL); (**C**) TET (0.125 μg/mL); and (**D**) CLA (0.125 μg/mL).

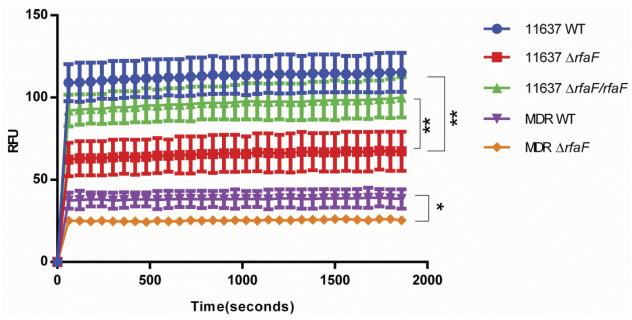

Figure 2 Comparison of EB accumulation in various types of *H. pylori* strains.
Notes: The data presented are the means ± standard errors of the means from three separate experiments. *$P<0.05$; **$P<0.01$.

susceptibility reduction.[12] We screened 10 clinical strains with relatively high MIC levels of AMO, TET, and CLA and 11,637 WT strain, and sequenced *rfaF*. After alignment analysis, although no deletions or insertions were found, there were conservative amino acid substitutions in 9 out of 10 clinical strains, and no mutations were found in 11,637 WT strain (Table 3). In addition, interestingly, K331R mutation type accounted for 44.44% of all mutants. Therefore, we conclude that the high mutation rate of the *rfaF* in clinical strains is an important factor causing resistance to AMO, TET, and CLA.

Discussion

Although the resistance to AMO and TET is low, the resistance rates of these two antibiotics and CLA have increased in recent years.[21–24] The *rfaF* (*waaF*) gene encodes a protein that transfers a second heptose residue and integrates it into the core of the LPS. Disruption of heptose transport results in a herpes-free phenotype called "deep roughness".[25] To evaluate the function of *rfaF*, we generated knockout strains from both 11,673 WT and MDR WT strain (No. FZ068) simultaneously. The results revealed that their Δ*rfaF* was cross-resistant to AMO, TET, and CLA. The role of *rfaF* in causing drug resistance is universal. In agreement with our results, previous studies have reported that *Escherichia coli rfaF* mutation reduced drug sensitivity to AMO and TET.[12] This may be due to the mutation that causes a decline in the expression of a drug-dependent pore protein or a transferring protein, resulting in the decrease of cell membrane permeability to the drug, thus decreasing the concentration of the drug in the cell. However, chloramphenicol susceptibility results are contradictory. The reason for this difference is that *rfaF* mutation led to the expression of chloramphenicol-dependent porins to be completely reversed. Our results show that compared with the MIC values of AMO, TET, CLA, and CHL of MDR WT strain, MDR Δ*rfaF* strain only increased by 0.5-fold. One of the possible reasons may be that the knockout strategy of MDR strain was incomplete, which retained a part of the ORF sequence, and this part may continue to express partially functional proteins. The other more likely reason is that *rfaF* in MDR may have been mutated before it was knocked out, so the *rfaF* protein activity may be partially inactivated. In line with our inference, the A341T mutation was found in the MDR *rfaF* strain by alignment analysis of amino acid conserved sequences. Coincidentally, in another study, the *rfaF* mutation in clinical *E. coli* isolates also occurred at position 341 where A was replaced by T to confer antibiotic resistance.[12] If this mutation can lead to a partial inactivation of the protein, then the basic drug MIC value of the MDR strain will increase. Consistent with our hypothesis, the results showed that the MICs of

Table 3 *rfaF* Amino Acid Mutation Analysis

Strains	MIC (μg/mL)					Putative Amino Acid Conserved Sequence Variations Of Reducing Sensitivity
	AMO	TET	CLA	MEM	LEV	
11,637	0.064	0.125	0.125	0.5	0.25	No found mutation
NO.FZ001	0.125	0.5	128	128	1	K331R
NO.FZ035	0.25	0.5	128	256	4	No found mutation
NO.FZ038	0.25	2	2	32	1	K331R
NO.FZ040	0.125	0.5	128	256	16	I59A, L345F
NO.FZ045	0.25	0.5	16	32	0.125	P192L, K331R
NO.FZ068	0.125	0.25	68	256	8	A341T
NO.FZ076	0.5	0.125	68	16	8	S250N
NO.FZ113	0.5	0.5	256	256	4	A276T
NO.FZ120	1	0.5	0.25	32	4	A342T
N0.FZ202	0.5	0.5	128	128	0.5	E198K, K331R

AMO, CLA, TET, and CHL of MDR WT strain were higher than that of 11,637 WT strain.

EB cumulative fluorescence was used to reflect drug permeability: the lower MIC value, the stronger the EB cumulative fluorescence intensity.[8,26] We found that the cumulative EB fluorescence intensity of MDR WT was lower than that of 11,637 WT, which is consistent with the result that the basic MIC values of AMO, TET, and CLA in MDR WT strain were higher than that of 11,637 WT strain. The cumulative fluorescence intensity of EB of Δ*rfaF* was significantly lower in 11,637 and in MDR than that of the WT strain, which was consistent with the significant increase in MIC levels of AMO, TET, and CLA of Δ*rfaF* strains. Our findings indicate that the higher the MIC level of the drug, the lower the EB cumulative fluorescence. A drug permeability mechanism study similar to ours indicates that TET-resistant strains reduce the accumulation of TET.[10] We conclude that the mechanism of reducing drug sensitivity of Δ*rfaF* is the decrease in drug permeability of the cell membrane.

This study discovered that *H. pylori rfaF* gene was directly related to drug sensitivity, and its mechanism of drug resistance was the decrease in drug permeability of the cell membrane. Interestingly, we found that in the clinical strains, *rfaF* amino acid conserved sequence had high mutation rate and the K331R mutation was the most frequent (44.44% of all mutations). This result suggests that *rfaF* confers resistance to drugs in clinical strains. The high mutation rate of *rfaF* accounted for the relatively high levels of MIC of AMO, TET, and CLA. Further analysis needs to be performed to confirm the active site of *rfaF* protein. In conclusion, this study provides a useful target gene for finding solutions to antibiotic resistance.

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Disclosure

The authors report no conflicts of interest in this work.

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