

Efflux pump gene *hefA* of *Helicobacter pylori* plays an important role in multidrug resistance

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CONCLUSION: The efflux pump gene *hefA* plays an important role in multidrug resistance of *H pylori*.

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Key words: Efflux pump; *Helicobacter pylori*; Multidrug resistance; Fluorescence real-time quantitative PCR; Knockout mutant

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Abstract

AIM: To determine whether efflux systems contribute to multidrug resistance of *H pylori*.

METHODS: A chloramphenicol-induced multidrug resistance model of six susceptible *H pylori* strains (5 isolates and *H pylori* NCTC11637) was developed. Multidrug-resistant (MDR) strains were selected and the minimal inhibitory concentration (MIC) of erythromycin, metronidazole, penicillin G, tetracycline, and ciprofloxacin in multidrug resistant strains and their parent strains was determined by agar dilution tests. The level of mRNA expression of *hefA* was assessed by fluorescence real-time quantitative PCR. A *H pylori* LZ1026 knockout mutant ($\Delta H pylori$ LZ1026) for (putative) efflux protein was constructed by inserting the kanamycin resistance cassette from pEGFP-N2 into *hefA*, and its susceptibility profiles to 10 antibiotics were evaluated.

RESULTS: The MIC of six multidrug-resistant strains (including 5 clinical isolates and *H pylori* NCTC11637) increased significantly (≥ 4 -fold) compared with their parent strains. The expression level of *hefA* gene was significantly higher in the MDR strains than in their parent strains ($P = 0.033$). A *H pylori* LZ1026 mutant was successfully constructed and the $\Delta H pylori$ LZ1026 was more susceptible to four of the 10 antibiotics. All the 20 strains displayed transcripts for *hefA* that confirmed the *in vitro* expression of these genes.

INTRODUCTION

H pylori is a Gram-negative bacterium that colonizes human gastric mucosa^[1] and plays an important role in the pathogenesis of chronic gastritis, peptic ulcer, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma^[2,3]. Successful treatment usually requires two or three kinds of antibiotics in combination with a proton pump inhibitor. In recent years, the eradication rate of *H pylori* for standard therapies has decreased due to the increasing antimicrobial resistance^[4,5]. Possible mechanisms of intrinsic drug resistance involve decreased drug uptake or increased drug efflux^[6]. Efflux of compounds is a phenomenon commonly observed in bacteria^[7-9]. Through this process, organisms are protected from possible toxic effects of metabolite accumulation or external compounds, and compound efflux results in a decreased susceptibility to a variety of antibiotics. Five families of multidrug efflux transporters have been described^[10]. One of them, widespread in Gram-negative bacteria, is the resistance-nodulation-division (RND) family of efflux systems^[11-13]. The RND family of efflux systems has three components: inner membrane efflux proteins (IEPs) which act with the other two components, a periplasmic efflux protein (PEP) which facilitates interaction with the other two components, and an outer membrane efflux protein (OEP) which

is the TolC (the outer membrane efflux protein in *Escherichia coli*) or a TolC homolog^[14].

Bina JE and coworkers^[15] have identified three RND efflux systems, namely *hefABC*, *hefDEF*, and *hefGHI*. Each of them is consisted a translocase, an accessory protein, and a TolC homolog. *hefA*, *hefD*, and *hefG* are the TolC homolog encoding the outer membrane efflux protein. In their study, the *hefGHI* operon was expressed only *in vivo*. Nevertheless, they could not establish a role of these efflux systems in antibiotic resistance. An unfortunate choice of compounds and a methodology that may not be sensitive enough to relatively small differences in susceptibilities between the mutant and parental strains may have impacted their study. van Amsterdam *et al*^[16] identified 27 putative translocases in the *H pylori* 26695 genome, but only four putative *H pylori* OEPs or TolC homologs. Parallel translocases may function in a limited number of OEPs^[17]. Thus, inactivation of a TolC-like protein may affect the functions of multiple translocases. They evaluated the susceptibility profiles of the inactivation of the four TolC homologs after insertion, but could not quantitate the operon expression levels of the efflux systems in multidrug resistant strains of *H pylori*. In view of the biological difference of the *H pylori* strains in Asia, Europe and North America^[18], the present study was to determine whether efflux systems of *H pylori* contribute to antimicrobial susceptibility. A chloramphenicol-induced multidrug resistance model of 6 susceptible *H pylori* strains (5 isolates and *H pylori* NCTC11637) was developed by detecting the minimal inhibitory concentration (MIC) of the strains to metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin. The levels of mRNA expression of *hefA* in multidrug-resistance (MDR) strains and their parent strains were assessed by fluorescence real-time quantitative PCR. To further evaluate the role of *hefA* in multidrug resistance of *H pylori*, a clinical isolate of *H pylori* LZ1026 was selected, which represent the Chinese strains. A *H pylori* LZ1026 knockout mutant was constructed and its susceptibility profiles to 10 kinds of antibiotics were evaluated.

MATERIALS AND METHODS

Reagents

Chemical reagents were purchased from TaKaRa Biotechnology Co. Ltd (Dalian, China). Restriction enzymes, polymerases, T4 DNA ligase, Quant SYBR Green PCR Kits and other molecular biology reagents were purchased from Biosail Biotechnology Co. Ltd (Beijing, China), and used following their manufacturers' instructions. Amoxicillin, cefotaxime, ceftazidime, polymyxin B, nalidixic acid, clarithromycin, norfloxacin, gentamicin, metronidazole, tetracycline, erythromycin, chloramphenicol, and ciprofloxacin, penicillin G were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Nucleic acid sequencing and synthesis were performed in SaiBaiSheng Biotechnology Co. Ltd (Beijing, China).

Bacterial strains, plasmid and culture conditions

H pylori LZ1026 and 20 other clinical isolates of *H pylori* were obtained from routine cultures of clinical gastric biopsies from patients with peptic ulcer or chronic active gastritis at the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). *H pylori* NCTC11637 obtained from HeNan Key Laboratory of Molecular Medicine was used as a reference. Isolates were cultured on Brucella agar medium plates containing 7% lysed sheep blood at 37°C under microaerobic conditions (50 mL/L O₂, 100 mL/L CO₂, 850 mL/L N₂) for 48-72 h. Identification of *H pylori* isolates was based on the results of Gram staining, cell morphology, and positive reaction for catalase, oxidase and urease activity. Putative multidrug efflux knockout mutant of *H pylori* LZ1026 was grown on sheep blood agar plates containing 25 µg of kanamycin (KAN) per mL and 100 µg of ampicillin (AMP) per mL. The vectors pBluescript II SK (-) and pEGFP-N2 were from HeNan Key Laboratory of Molecular Medicine. *E. coli* DH5α was used for subcloning the *hefA* operon. *E. coli* were grown in Luria broth. For cloning in *E. coli*, 100 mg/mL AMP was used.

Induction of multiple antibiotic resistances of clinical isolates

Susceptible strains were isolated from gastric biopsy samples using multiple antibiotic resistance induction tests. Induction of chloramphenicol resistance of susceptible isolates was performed by selecting resistant colonies that arose in the agar plates containing 1/2 × MIC chloramphenicol. The resistant colonies were incubated for 48-72 h under microaerobic conditions to increase the concentration of chloramphenicol by one fold up to 128 × MIC. The strains were further incubated on fresh plates with no chloramphenicol for 4 generations, and then transferred onto plates containing 4 × MIC chloramphenicol^[19].

Induced colonies were maintained on plates containing 4 × MIC of erythromycin, metronidazole, tetracycline, penicillin G or ciprofloxacin, respectively. The colonies were incubated for 48-72 h under microaerobic conditions. A four-fold increase in MIC of the multidrug resistant strains was considered significant^[20]. Six multidrug resistant strains were selected.

Assessment of susceptibilities to antimicrobials

H pylori cells grown for 48 h on sheep blood agar plates were resuspended in phosphate-buffered saline. Suspensions of *H pylori* were adjusted to an optical density of 0.1 at 625 nm, and 1 µL of these suspensions containing approximately 10⁵ CFU/mL, was spread on horse blood agar plates containing approximately 5 × 10⁴ bacteria within 5 mm^[21]. MICs of erythromycin, metronidazole, penicillin G, tetracycline, and ciprofloxacin in multidrug resistant strains and their parent isolates were determined with the conventional two-fold agar dilution tests. MICs of the kinds of 10 antibiotics against the *hefA* mutant strain of *H pylori* LZ1026 and its wild strain were also detected^[22].

Real-time RT-PCR

RNA was isolated using the total RNA kit (SBS Genetech Co., Ltd Beijing, China) and reverse transcribed into cDNA. Complete removal of DNA was verified by direct PCR with the RNA as a template (Figure 1). *hefA*, versus *gyrB* (a housekeeping gene encoding for gyrase B), was utilized to study the relative expression of the *hefA* gene in 6 multidrug resistant strains, and their parent strains: *H pylori* 03154, *H pylori* 12025, *H pylori* 12021, *H pylori* 11032, *H pylori* 03174, NCTC11637. cDNA of *hefA* and *gyrB* was amplified using a 5700 sequence detector real time PCR machine (Perkin Elmer Company) in the presence of Real Master Mix (SYBR Green). The gene-specific primers used were designed based on the sequence alignments of the genes from *H pylori* 11637 in GenBank. The sequences of *hefA* (accession No: AF059041) are F: (5'-ACGCCTCGAGTAAAAGCGCAAGGGAATTTG-3') and R: (5'-ACGCTCTAGATTTCGCTAATTGGCCTAGCAT-3'). The PCR primers were predicted to amplify a 162-bp amplicon. Expression of the housekeeping gene *gyrB* (accession No: AB084049) was assessed in parallel with the primer pair *gyrB*: F: (5'-TTACTACGACTTATCTGGGGCTAGCGCTG-3') and R: (5'-CCCATCAATTTCCACAT TCTCCGC-3'). The PCR primers were predicted to amplify a 267-bp amplicon.

To plot standard curves for real-time PCR, cDNAs of the *hefA* and *gyrB* genes were cloned into the pMD19-T vector system (TaKaRa Biotechnology). The vector containing cDNAs of the *hefA* or *gyrB* genes with known molar concentrations was utilized. Single reactions were prepared for each cDNA along with each serial of dilution using the SYBR Green Master Mix (Stratagene). Each PCR also included a reverse transcription negative control to confirm the absence of genomic DNA, and a non template negative control to check the primer-dimer. Each reaction consisted of 20 μ L, containing 2 μ L of cDNA and 5 pmol of each primer. The cycling conditions were 1 cycle of denaturation at 95°C for 1 min, followed by 35 three-segment cycles of amplification at 95°C for 30 s, at 55°C for 1 min, and at 68°C for 30 s during which the fluorescence was automatically measured, and one three-segment cycle of product melting at 95°C for 1 min, at 55°C for 30 s, and at 95°C for 30 s. The baseline adjustment method of the Gene Amp 5700 SDS (Perkin-Elmer) software was used to determine Ct (the threshold cycle) in each reaction. A melting curve was plotted for each primer pair to verify the presence of one gene-specific peak, and the absence of primer dimer. All samples were amplified in three independent reactions, and the mean was used for further analysis^[23-25].

To assess whether the differences were actually statistically significant, an analysis of paired-samples *t* - test was utilized to study the degree of statistical significance of *hefA* gene expression between MDR and parent strains.

Mutagenesis of putative efflux operon

All standard DNA techniques, transformation of *E.coli*, and DNA analysis procedures were performed as

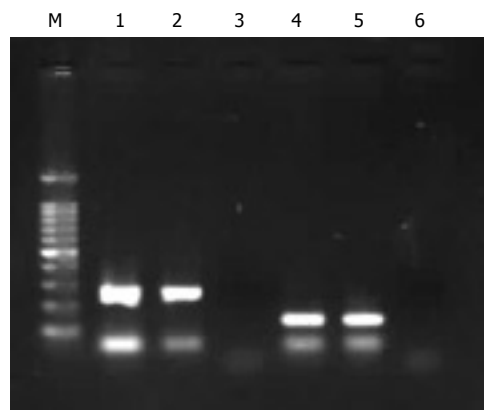


Figure 1 RT-PCR products of the *hefA* and *gyrB* genes. M: 100 bp DNA Marker; lanes 1 and 2: PCR products of *gyrB*; lanes 4 and 5: PCR products of *hefA*; lanes 3 and 6: PCR products with RNA as a template.

previously described^[26-28]. Plasmid DNA was isolated using a TIANprep Mini plasmid kit (TianGen Biotech Co. Ltd, Beijing, China). *H pylori* LZ1026 strain was isolated from a patient with peptic ulcer, and its internal portion (bp 277 to 1 574) of the *hefA* gene (reference from *H pylori* 11637 in GenBank accession number AF059041) was amplified. A restriction fragment, approximately 1298 bp, was purified and ligated into XbaI and XhoI-digested vector pBluescript II SK(-). *H pylori* LZ1026 knockout mutant for (putative) efflux protein was constructed by inserting the KAN resistance cassette from vector pEGFP-N2 into *hefA*, resulting in an 859 bp deletion. This suicide plasmid was then introduced into *H pylori* LZ1026 by natural transformation, and transformants were selected by plating them on a selective medium containing 25 mg of kanamycin/mL. Insertion of the KAN resistance cassette at the desired location(s) in the *H pylori* putative efflux gene was confirmed by PCR. Each clone was sequenced by Biosia Biotechnology Company (Shanghai, China).

RESULTS

Phenotypic induction of *hefA*-mediated resistance of *H pylori*

To obtain the multidrug-resistant strains, a chloramphenicol-induced model was developed. Following one-fold concentration increase of chloramphenicol, the MICs of chloramphenicol-induced strains resistant to metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin were detected. The MICs of 6 multidrug-resistant strains (including 5 clinical isolates and *H pylori* NCTC11637) were significantly increased (\geq 4-fold) compared with their parent strains (Table 1).

Expression of *hefA* in multidrug resistant strains (MDR) and their parent isolates (PT)

The expression of *hefA* in multidrug resistant strains and their parent isolates was assessed by relative real-time RT-PCR. Each relative expression value was the mean of three replicas. The relative expression of *hefA* versus *gyrB*

Table 1 Chloramphenicol-induced multidrug resistance profiles of *H pylori* strains

Strains	Exptl condition	Agar dilution test MIC ($\mu\text{g/mL}$)				
		MTZ	ERY	CIP	TET	PEN
03154	Before induction	0.125	0.125	0.25	0.25	0.125
	After induction (fold)	8 \times	4 \times	4 \times	8 \times	16 \times
12025	Before induction	2	0.063	0.125	0.03	0.063
	After induction (fold)	16 \times	2 \times	8 \times	4 \times	16 \times
12021	Before induction	4	0.125	0.25	0.125	0.063
	After induction (fold)	16	1 \times	16 \times	8 \times	8 \times
11032	Before induction	1	0.063	0.063	0.063	0.25
	After induction (fold)	8 \times	4 \times	4 \times	16 \times	16 \times
03174	Before induction	0.5	0.125	0.25	0.125	0.125
	After induction (fold)	8 \times	1 \times	8 \times	4 \times	32 \times
11637	Before induction	0.5	0.063	0.125	0.03	0.25
	After induction (fold)	4 \times	2 \times	4 \times	8 \times	16 \times

MTZ: Metronidazole; ERY: Erythromycin; CIP: Ciprofloxacin; TET: Tetracycline; PEN: Penicillin G.

Table 2 Relative expression of *hefA* in PT and MDR strains

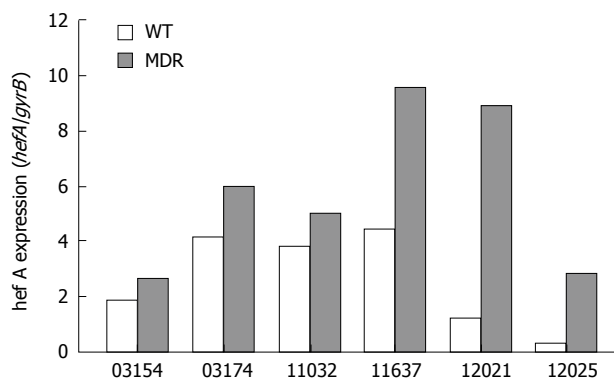
Strains	PT (<i>hefA/gyrB</i>)	MDR (<i>hefA/gyrB</i>)
03154	1.87	2.67
12025	0.32	2.84
12021	1.22	8.94
11032	3.84	5.04
03174	4.16	6.03
11637	4.41	9.56
Total (mean \pm SD)	2.6356 \pm 1.7245	5.8466 \pm 2.9370

PT: Parent isolates; MDR: Multidrug resistant strains.

in 5 clinical isolates and *H pylori* 11637 was significantly higher in MDR (5.8466 \pm 2.9370) than in PT (2.6356 \pm 1.7245) (Table 2). The results reveal that the relative expression of *hefA* was higher in the induced multidrug resistant strains than in their wild isolates. The difference in *hefA* expression was statistically significant ($P = 0.033$) (Figure 2).

Active efflux systems in *H pylori*

To further evaluate the role of efflux pump gene *hefA* of *H pylori* in multidrug resistance, a *H pylori* LZ1026 mutant was successfully constructed, and its disrupted *hefA* gene was uniquely different from that of the wild strain. A *hefA* gene fragment (about 1147 bp) of *H pylori* LZ1026 was sequenced and submitted to the GenBank (accession No. EU271683). Susceptibilities to wild-type strain of *H pylori* (*H pylori* LZ1026) and its isogenic mutant with putative RND outer membrane efflux protein were determined. The *hefA* (*H pylori* 0605, numbers refer to the loci in *H pylori* 26695) was genetically inactivated. MICs were determined by agar dilution tests. The *H pylori* mutant increased its susceptibility. The MIC of four of the 10 tested kinds of antibiotics (amoxicillin, cefotaxime, ceftazidime, polymyxin B, ciprofloxacin, nalidixic acid, clarithromycin, norfloxacin, chloramphenicol, and gentamicin,) was decreased at least 8-fold in the *H pylori* LZ1026 mutant, 8-fold in clarithromycin, 32-fold in cefotaxime, 16-fold

**Figure 2** Expression of the *hefA* gene in PT and MDR strains.

in chloramphenicol, 8-fold in gentamicin. The MICs were decreased after treatment with different antibiotics, which is consistent with the hypothesis that the product of *hefA* participates in multidrug efflux.

Transcribed gene encoding TolC homolog in vitro

Transcription of the gene *hefA* encoding a homolog of *E. coli* TolC outer membrane protein was assessed by RT-PCR with cDNA obtained from 20 different *H pylori* isolates. All the 20 strains displayed transcripts of *hefA* that confirmed the *in vitro* expression of these genes (data not shown).

DISCUSSION

Treatment of *H pylori* infection usually requires administration of two or more kinds of antibiotics concurrently. However, it may ultimately not be sufficient due to increased antibiotic resistance. Indeed, bacterial resistance to antibiotics hampers treatment of *H pylori* infections. Besides chromosomally-encoded drug resistance, intrinsic resistance to toxic compounds through increased export might be of importance in multidrug resistance of *H pylori*. Efflux systems have been identified in *H pylori*^{15,16}, yet the possibility that these systems are implicated in multidrug resistance has not been established previously¹⁵. In this study, the *hefA* gene was chosen from three TolC homologs: *hefA*, *hefD* and *hefG*, which encode the outer membrane efflux protein in *H pylori*. It was demonstrated earlier that the *hefD* and *hefG* genes are only poorly expressed *in vitro* and only RND efflux systems are known to have a wide variety of antibiotic substrates^{15,22}.

In the present study, because it was laborious to select the multidrug resistant strains from clinical isolates of *H pylori*, we developed a chloramphenicol-induced multidrug resistant model of *H pylori*, for the first time to our knowledge. Using this model, six multidrug resistant strains were elicited successfully, and their MICs of metronidazole, tetracycline, erythromycin, penicillin G, and ciprofloxacin were significantly increased (≥ 4 -fold) compared with their parent strains, except that the MIC of erythromycin was mildly increased in 4 wild strains, indicating a difference in substrate specificity with other bacteria^{29,30}. In contrast, they could only dampen the

function of a group of efflux systems by constructing mutant strain. But, they could not quantitate the operon expression levels in the efflux systems^[16]. In this study, the relative real-time RT-PCR was introduced to assess the expression level of the *hefA* gene encoding the outer membrane efflux protein in 5 clinical isolates and *H pylori*11637, suggesting that the relative expression of *hefA* is higher in induced multidrug resistant strains than in their wild susceptible isolates. This is consistent with the hypothesis that the expression of RND efflux systems in many bacteria is regulated by environmental stimuli, including the presence of antibiotics and antimicrobial compounds, growth stage, and stress factors^[7-9]. By inserting the KAN resistance cassette into *hefA*, we also constructed a *H pylori* mutant strain ($\Delta H pylori$ LZ1026). By detecting the susceptibility to 10 kinds of antimicrobials with a more sensitive method of two-fold agar dilution tests, we showed that similar to other gram-negative organisms, the *H pylori hefA* mutant increased its susceptibility to antibiotics, which is consistent with the findings in a previous study^[22], confirming that high expression of the *hefA* plays an important role in MDR of *H pylori*. Since *H pylori* contain an active multidrug efflux mechanism, compound efflux needs to be taken into account when determining resistance mechanisms in *H pylori*.

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COMMENTS

Background

In recent years, the eradication rates of *H pylori* for standard therapies have decreased due to increasing antimicrobial resistance. Multidrug resistant *H pylori* strains have already been discovered. Possible mechanisms underlying multidrug resistant *H pylori* strains are involved in decreased drug uptake or increased drug efflux. Efflux of compounds is a phenomenon commonly observed in bacteria, and compound efflux decreases the susceptibility of *H pylori* strains to a variety of antibiotics. The contribution of efflux proteins to antibiotic resistance of *H pylori* strains is not well established.

Research frontiers

With the increasing antimicrobial resistance to *H pylori*, multidrug resistant *H pylori* strains have been discovered. Researches on the mechanisms of antibiotic resistance of *H pylori* have becoming a medical research hotspot. The mechanism underlying multidrug resistance of *H pylori* is still unclear.

Innovations and breakthroughs

The contribution of efflux proteins to antibiotic resistance of *H pylori*, strains is not well established. In view of the biological difference of *H pylori* strains in Asia, Europe and North America, *H pylori* strains were isolated from Chinese people in the present study. The study was to determine whether efflux systems in *H pylori* strains contribute to their antimicrobial susceptibility. A chloramphenicol-induced multidrug resistant model of *H pylori* strains was developed. The level of mRNA expression of *hefA* in multidrug-resistant (MDR) strains and their parent strains were assessed by fluorescence real-time quantitative PCR. A *H pylori* LZ1026 knockout mutant was constructed and its susceptibility profiles to 10 kinds of antibiotics were evaluated.

Applications

This study confirmed that high expression of the *hefA* plays an important role in MDR of *H pylori*. Since *H pylori* contain an active multidrug efflux mechanism,

compound efflux needs to be taken into account when determining resistance mechanisms in *H pylori*.

Terminology

Efflux pump is chromosomally located or acquired by bacteria, which can either be activated by environmental signals or by a mutation in a regulatory gene. Two major categories exist: one is energized by proton motive force and the other is dependent on ATP. The pumps may have limited or broad substrates, the so-called multiple drug resistant pumps themselves form a number of related families.

Peer review

The authors studied the significance of efflux pump gene *hefA* in multidrug resistance of *H pylori*. The study is interesting and well designed.

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