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幽門桿菌對 Clarithromycin 抗藥機制之研究

Molecular Mechanisms of Clarithromycin Resistance in *Helicobacter pylori*

主持人:王錦堂　　國立台灣大學醫學院內科

一、中文摘要

利用聯合抗生素療法消除幽門螺旋桿菌(*Helicobacter pylori,Hp*)已成為治療消化性胃潰瘍疾病的標準方法,開羅黴素(Clarithromycin,Cla)是最近廣為接受,用以消除幽門螺旋桿菌的抗生素.然而,幽門螺旋桿菌對開羅黴素抗藥性之產生,使得包含開羅黴素治療法的效果大打折扣.因此,我們試圖根據其他細菌,先前已發表對Macrolides 這類抗生素的抗藥機制,來研究幽門螺旋桿菌產生開羅黴素抗藥性之可能的機制.由退化的寡核甘酸引子(Degenerate Primers),進行聚合脢連鎖反應(Polymerase Chain Reaction),無法找出相對應於其他細菌之甲基酵素(Methylase),藥物去活性酵素(Drug Inactivation Enzyme)或排出幫浦蛋白質(Efflux Pump Proteins).

利用一株具開羅黴素抗藥性之幽門螺旋桿菌的 Genomic DNA,作成表現基因庫(Expression Library),送到大腸桿菌(*E. coli*)細胞中,進行篩選.經由再次轉型作用(Retransformation),可得到六株對開羅黴菌具有抗藥性的菌株.由南方轉漬實驗(Southern blot)及核酸定序(DNA Sequencing)的結果得知,其中四個帶有一個相同的基因,比較其核酸及氨基酸序列,顯示此1.3kb片段和枯草桿菌(*Bacillus subtilis*)之 3-oxoadipate CoA-tranferase subunit A(*yxiD*)及 subunit B(*yxiE*)基因具有高度相似性.然而,由開羅黴素去活性分析(Chlarithromycin Inactivation Assay)及Knock-out Mutant Analysis,顯示此基因可增加對開羅黴素的抗藥性,但只限於大腸桿菌,並不存於幽門螺旋桿菌中.

另一方面,分析由臨床分離,對開羅黴素具抗藥性之幽門螺旋桿菌,其 23S rRNA 基因序列,顯示在核甘酸第 2515 位置上有A變為G的 transitional mutation 存在.利用自然轉形作用(Natural Transformation),送入對開羅黴素具抗藥性的 23S rRNA 基因,可將原本對開羅黴素敏感的幽門螺旋桿菌變為具抗藥性.

因此,我們得到一個結論:23S rRNA 基因突變,是幽門螺旋桿菌對開羅黴素產生抗藥性之充分且為主要之抗藥機制.分析在台灣分離得到之抗開羅黴素的幽門螺旋桿菌,其 23S rRNA 基因,第 2515 個核甘酸包含著 A 變為 G 的突變.3-oxoadipate CoA-transferase subunit A(*yxjD*)基因的大量表現,可在大腸桿菌中提昇對開羅黴素的抗藥性,但在幽門螺旋桿菌中,其對開羅黴素之抗藥性似乎不具有顯著的影響.

關鍵字:幽門螺旋桿菌、開羅黴素、抗藥性、抗生素

Abstract:

subunit A(*yxiD*)

Combination antibiotic therapy for *Helicobacter pylori* has now become the standard means of treating peptic ulcer diseases. Clarithromycin is a newly adopted antibiotic for *H. pylori* eradication. However, resistance to clarithromycin reduces the efficacy of clarithromycin-containing regimens. We explored mechanisms of clarithromycin resistance by evaluation *H. pylori* for macrolide resistance mechanisms reported in *H. pylori* and other bacteria. Degenerate polymerase chain reaction analysis of the *H. pylori* genome failed to yield products homologous to methylase, a drug inactivation enzyme, or efflux pumps. Clarithromycin selection in *Escherichia coli*NM522, transformed with an expression library that was constructed with genomic DNA from a clarithromycin-resistant strain of *H. pylori*, revealed six clones that conferred clarithromycin resistance consistently after retransformation. Southern hybridization and DNA sequencing revealed that four of the six clones that contained the same locus. Comparison of DNA and amino acid sequences showed that the 1.3-kb DNA fragment had significant homology to the 3-oxoadipate CoA-transferase subunit A(*yxiD*) and subunit B(*yxiE*) of *Bacillus subtilis*. However, the clarithromycin inactivation assay and knockout mutation analysis showed that the gene increases clarithromycin resistance in *E. coli*, but no in *H. pylori*. In contrast, sequencing of the 23S rRNA gene in six clarithromycin-resitant *H. pylori* clinical isolates revealed an A to G transitional mutation at position 2515 of the 23S rRNA gene in all isolates. Natural transformation with the 23S rRNA gene from resistant strains conferred clarithromycin resistance in clarithromycin-sensitive strains. We conclude that the 23S rRNA mutation is sufficient to confer clarithromycin resistance and that it is the major mechanism of clarithromycin resistance in *H. pylori*.

Keywords: *Helicobacter pylori*, drug resistance, antibiotic, clarothromycin, 23S rRNA gene, 3-oxoadipate CoA-transferase

Molecular Mechanisms of Clarithromycin Resistance in *Helicobacter pylori*

Pei-Fang Hsieh,[1] *Jyh-Chin Yang,*[2] *Jaw-Town Lin,*[2] *and Jin-Town Wang*[1,2]

Abstract: Combination antibiotic therapy for *Helicobacter pylori* has now become the standard means of treating peptic ulcer diseases. Clarithromycin is a newly adopted antibiotic for *H. pylori* eradication. However, resistance to clarithromycin reduces the efficacy of clarithromycin-containing regimens. We explored mechanisms of clarithromycin resistance by evaluating *H. pylori* for macrolide resistance mechanisms reported in *H. pylori* and other bacteria. Degenerate polymerase chain reaction analysis of the *H. pylori* genome failed to yield products homologous to methylase, a drug inactivation enzyme, or efflux pumps. Clarithromycin selection in *Escherichia coli* NM522, transformed with an expression library that was constructed with genomic DNA from a clarithromycin-resistant strain of *H. pylori*, revealed six clones that conferred clarithromycin resistance consistently after retransformation. Southern hybridization and DNA sequencing revealed that four of the six clones contained the same locus. Comparison of DNA and amino acid sequences showed that the 1.3-kb DNA fragment had significant homology to the 3-oxoadipate CoA-transferase subunit A (*yxjD*) and subunit B (*yxjE*) of *Bacillus subtilis*. However, the clarithromycin inactivation assay and knockout mutation analysis showed that the gene increased clarithromycin resistance in *E. coli*, but not in *H. pylori*. In contrast, sequencing of the 23S rRNA gene in six clarithromycin-resistant *H. pylori* clinical isolates revealed an A to G transitional mutation at position 2515 of the 23S rRNA gene in all isolates. Natural transformation with the 23S rRNA gene from resistant strains conferred clarithromycin resistance in clarithromycin-sensitive strains. We conclude that the 23S rRNA mutation is sufficient to confer clarithromycin resistance and that it is the major mechanism of clarithromycin resistance in *H. pylori*.

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Key words:
Helicobacter pylori
drug resistance
antibiotic
clarithromycin
23S rRNA gene
3-oxoadipate CoA-transferase subunit A (*yxjD*)

Helicobacter pylori, a spiral Gram-negative bacterium, was first isolated in 1982 from the gastric mucosa of a patient with gastritis and peptic ulcer [1]. Studies have shown that eradication of *H. pylori* infection results in cure of peptic ulcer diseases [2, 3] and regression of gastric mucosa-associated lymphoid tissue lymphoma (MALToma) [4]. Therefore, antibiotic therapy to eradicate *H. pylori* infection has been recommended in treating patients with peptic ulcer diseases and MALToma. *H. pylori* is sensitive to a wide range of antibiotics *in vitro*, including penicillin, nitroimidazoles, cephalosporins, fluoroquinolones, and macrolides [5–9]. In clinical practice, a single antibiotic given alone is relatively ineffective in eradicating this organism. Eradication rates with clarithromycin have been greater than with any other individual antibiotic [10], but the emergence of clarithromycin-resistant *H. pylori* strains has been reported in several studies (1–8% of cases), and can result in treatment failure [11–13]. The resistance rate in Taiwan is low (approximately 3%, authors'

[1]Graduate Institute of Microbiology, College of Medicine, National Taiwan University, [2]Department of Internal Medicine, National Taiwan University Hospital, Taipei.
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Reprint requests and correspondence to: Dr. Jin-Town Wang, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, 1, Section 1, Jen-Ai Road, Taipei, Taiwan.

unpublished data). However, with the widespread application of clarithromycin, the resistance rate is expected to rise in the future. Understanding the mechanism of drug resistance would be helpful in coping with this clinical problem.

The mechanisms of resistance to clarithromycin-related macrolides have been studied in other bacteria and appear to include: 1) modification of the drug target via single nucleotide mutations [14] or post-transcriptional methylation of the 23S rRNA [15, 16]; 2) antibiotic modification by the bacteria [17–21]; and 3) active efflux of antibiotic [22, 23]. A to G and A to C mutations in both nucleotides 2514 and 2515 of the 23S rRNA gene have been observed in resistant *H. pylori* strains in Western countries [24, 25].

In this study, we explored the mechanisms of clarithromycin resistance in *H. pylori* by attempting to identify genes encoding methylase macrolide efflux mediators, or inactivating enzymes by degenerate polymerase chain reaction (PCR); attempting to isolate a novel gene for macrolide resistance, by antibiotic selection of *E. coli* strains transformed with an *H. pylori* expression library; and by sequencing and mutagenesis of the 23S rRNA gene in clarithromycin-resistant and -sensitive *H. pylori* strains.

Materials and Methods

Beginning in 1990, patients who underwent endoscopic examination at National Taiwan University Hospital were recruited for this study. Biopsy specimens from the gastric body and antrum were taken from patients with gastritis and peptic ulcer disease for bacterial culture. A total of 200 isolates were collected during the study period and tested for clarithromycin minimum inhibitory concentrations (MICs).

Bacteria were grown under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) at 37°C in brucella broth containing 5% fetal calf serum (FCS, Biological Industries, Kibbutz Beit Haemek, Israel). The strains were stored at –80°C after successful culture and drug sensitivity tests.

Three ATCC strains (ATCC 43526, ATCC 49503, ATCC 51932), obtained from the American Type Culture Collection (Manassas, VA, USA), and a Canadian strain, UA802 (gift from Dr. D.E. Taylor, University of Alberta, Edmonton, Canada), were used for comparison.

E. coli strains were used in the construction of a genomic DNA library and transformant selection. The strains were XLOLR (Δ(*mcrA*) *183* Δ(*mcrCB- hsd SMR-mrr*) *173end A1 thi-1 recA1gyrA96 relA1Lac* [F'*proAB lacI^q^* *ZΔM15* Tn*10*(Tet^r)]^c Su$^-$ (nonsuppressing) λ^r (lambda resistant)^l) (Stratagene, La Jolla, CA, USA); XL1-Blue MRF' (Δ(*mcrA*) *183*Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *pro AB lacI^q^ ZΔM 15* Tn*10* (Tet^r)]^c) (Stratagene); and NM522 (*supE thi1-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)*5*(rk-mk-) [F'*proAB lacI^q^ZΔM15*]) (Stratagene).

In vitro tests for drug sensitivity

The MICs of antibiotics in *E. coli* and *H. pylori* were determined by E-test (AB Biodisk, Solna, Sweden) and agar dilution methods, as described previously [26].

Degenerate primers and PCR

Degenerate primers were designed from genes reported to be associated with macrolide resistance in other bacteria (Table 1). Degenerate PCR was performed using USB Taq DNA polymerase (Amersham, Cleveland, OH, USA) under the following conditions: DNA was denatured for 3 minutes at 94°C, and then subjected to 35 amplification cycles consisting of elongation at 72°C for 90 seconds, denaturation at 94°C for

Table 1. Degenerate primers used to detect macrolide resistance determinants

Mechanism	Genotype	Primers		Reference
Target modification	erm	ermR	5'-GAAATHGGNNNNGGNAAAGGNCA-3'	15, 16
		ermL	5'-AAYTGRTTYTTNGTRAA-3'	
Drug inactivation	ereB	ereBR	5'-GACAAATACATGGCAGATTCTGTG-3'	17–21
		ereBL	5'-TATTCCATCGAAAGCTTTCTCGAG-3'	
	mphA	mphAR	5'-ATGACCGTAGTCACGACCGCCGA-3'	
		mphAL	5'-AGCTGCGCCTTCGCCGCAGCGA-3'	
	satA	satAR	5'-GTTGGAVRATACTCATATTAT-3'	
		satAL	5'-AGCAGGATTTCCTCCARCDAT-3'	
Active efflux	rbsA	rbsAR	5'-AAYGGCGCGGGYAARWSCACC-3'	22, 23
		rbsAL	5'-GGTVRGYTCRTCVAGSAKCAA-3'	

30 seconds, and annealing at 55°C, 42°C, or 37°C for 1 minute. The final elongation step was performed at 72°C for 10 minutes [27].

Construction and screening of the expression library

Chromosomal DNA from the clarithromycin-resistant *H. pylori* strain (MIC = 16–18 μg/mL), NTUH-CR1, was partially digested with the restriction endonuclease *Sau*3AI. DNA fragments 1 to12 kb in size were harvested from 0.8% agarose gels with the Geneclean III kit (Bio 101, La Jolla, CA, USA) and ligated into a *BamHI*-digested and alkaline-phosphatase-treated expression vector, λ-ZAPII (Stratagene) [28]. The recombinant λ-ZAPII plasmids were packaged into lambda phage particles and used to infect *E. coli* XL1-Blue MRF. A filamentous helper phage was then added to excise the pBK-CMV phagemid in *E. coli* XLOLR. Total phagemids were extracted and transformed into the *E. coli* strain NM522, which showed relatively low clarithromycin resistance (MIC = 50 μg/mL). Six phagemid clones with consistently high clarithromycin resistance were analyzed.

Southern blot analysis

Southern blot analysis was performed as described previously [29]. Briefly, DNA restriction fragments fractionated by agarose gel electrophoresis were transferred to nitrocellulose membranes and hybridized at 42°C with an enhanced chemiluminescent (ECL)-labeled probe (Amersham). Hybridization was revealed by autoradiography with Kodak XAR-OMAT film (Kodak, Rochester, NY, USA).

Antibiotic inactivation assay

E. coli strains containing the target DNA insert were grown overnight in LB broth containing kanamycin 50 μg/mL at 37°C with shaking. An overnight broth culture was diluted 10-fold with fresh broth and incubated for 2 hours at 37°C with shaking until the exponential growth phase was reached (OD_{600} = 0.3–0.5). Clarithromycin (60 μg/mL) was then added. Incubating with 1 mmol/L isopropyl thiogalactose for 3 hours induced expression of the target gene. The bacteria were then incubated overnight at 37°C. Clarithromycin was recovered by collecting the supernatant after centrifugation. MICs of the recovered clarithromycin were measured with the agar dilution test and compared with that of untreated clarithromycin.

Natural transformation of *H. pylori*

Natural transformation was performed by inoculating 100 μL of recipient *H. pylori* cells from the –80°C stock culture onto a cold Columbia blood agar plate, and incubating for 2 days at 37°C [30]. A heavy loopful of cells was then scraped from the plate and spread onto a cold plate in a diameter of 8 to 10 mm. Incubation was continued for 5 hours. We then spotted 10 to 20 μL of DNA (0.5–2 μg) in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA) directly onto the inoculated area (the bacterial lawn). The plate was incubated for 16 to 24 hours under microaerophilic conditions and the transformed cells were streaked onto Columbia blood agar plates containing selective antibiotics. The transformants were grown for 3 to 4 days.

Knockout mutants

A recombinant clone was constructed by inserting a chloramphenicol acetyltransferase (CAT) gene into a *SmaI* restriction site of the 3-oxoadipate CoA-transferase gene (*yxjD*) (Fig.1). The plasmid was transformed into wild type clarithromycin-resistant and -sensitive *H. pylori* isolates by natural transformation. Knockout mutants were selected with 20 μg/mL of chloramphenicol and confirmed by PCR and Southern blot analysis [31].

H. pylori 23S rRNA amplification and sequencing

PCR was performed under conditions described elsewhere [32]. The *H. pylori* 23S rRNA sequence from the Genbank Database [33] was used to design oligonucleotide primers (23SR: 5'-GCGTTGAATTGAAGCCCGAG-3' and 23SL: 5'-TGTGTGCTACCCAGCGATGC-3') that would allow the amplification of an 850-bp fragment of the 23S rRNA gene. The 850-bp fragment was purified from the PCR-amplification reaction using the Geneclean III kit. The purified DNA was then used as the template for the sequencing reaction with the Taq Dyedeoxy Terminator Cycle Sequencing Kit (Perkin Elmer, Foster City, CA, USA).

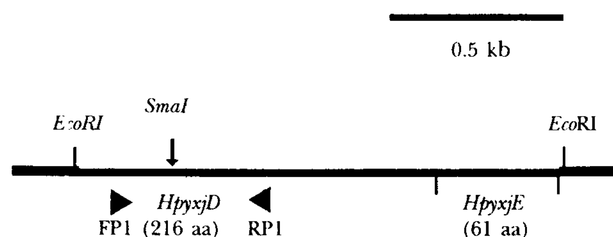

Fig.1 *Diagram of a plasmid clone (PCRII-Cla^R-CAT) containing the yx operon. Locations of the genes* hpyxjD *and* hpyxjE *are shown. A SmaI restriction site was used for the creation of the knockout mutants. Arrowheads represent the corresponding regions of individual primers (as named) whose sequences are given in the text.*

Mutagenesis

A 23S rRNA gene fragment was amplified by primers 23SR and 23SL, using NTUH-CR1 as the template DNA. PCR products (850 bp) were introduced into clarithromycin-sensitive *H. pylori* strains by natural transformation [34]. Wild-type 23S rRNA-gene fragments as well as a buffer without DNA were used as the negative controls. Mutants were selected from plates with various concentrations of clarithromycin. Chromosomal DNA from clarithromycin-resistant mutants was extracted and nucleotide position 2515 of the 23S rRNA was sequenced by PCR and direct sequencing.

Results

E. coli NM522 isolates harboring recombinant phagemids were tested for their ability to grow on LB plates supplemented with clarithromycin. Six strains were found to have grown better than strains with the pBK-CMV vector only on LB plates containing high concentrations of clarithromycin (Table 2). Southern blot analysis and direct sequencing revealed that four of the six phagemids harbored identical inserts (Fig. 2). All four shared a common *Sau3AI* fragment of 1.3 kb in size. DNA sequencing analysis showed that the 1.3-kb fragment had significant similarity (64%) with 3-oxoadipate CoA-transferase subunit A *(yxjD)* and subunit B *(yxjE)* from *Bacillus subtilis* (Fig. 3) [34, 35].

Because the homologue of the gene is unlikely to be an efflux pump, an antibiotic inactivation assay was done. The gene product failed to show clarithromycin inactivation activity (data not shown). Knockout mutants were created by inserting a CAT cassette (~ 1 kb) into the *SmaI* site of the *H. pylori yxjD* gene followed by

Table 2. Minimum inhibitory concentrations of clarithromycin in *Escherichia coli* NM522

	Strain	Clarithromycin (μg/mL) 50	60	80
No induction with IPTG	Clone 4	+	–	ND
	Clone 5	+	+	±
	Clone 6	++	++	±
Induction with IPTG	Clone 1	++	++	ND
	Clone 2	+	+	ND
	Clone 3	++	++	ND
pBK-CMV without insert		–	–	–

IPTG = isopropyl thiogalactose; ND = not determined; ++ = numerous colonies; + = 200–400 colonies; ± = poor growth; – = no growth.

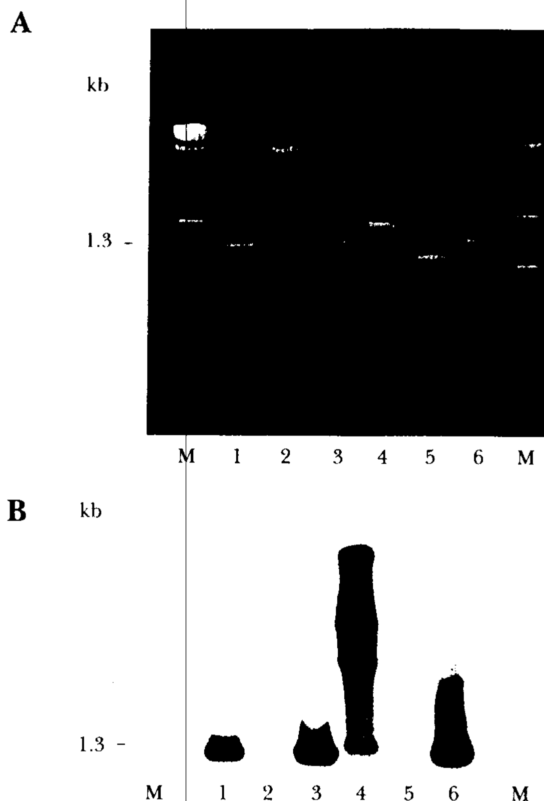

Fig. 2 *A) Gel electrophoresis and B) Southern blot analysis of six clarithromycin resistant phagemid clones in* Escherichia coli *NM522 strains after retransformation and clarithromycin selection. Lane M: molecular size markers. Lanes 1–6: six clones that were able to confer clarithromycin resistance after retransformation. DNA insert of clone 6 (Lane 6) was used as the probe for Southern blot analysis.*

natural transformation of wild-type *H. pylori* strains (Fig.4). Knockout mutants were confirmed by PCR with primers FP1 and RP1 (FP1: 5'-TGCAAGCGG-CGCTAAGAT-3' and RP1: 5'-CATCTGGGTCCA-ATTCCCCG-3') and Southern blot analysis (Fig. 5). However, the MICs did not change in knockout mutants as compared to the wild-type strains (Table 3).

To determine whether mutations in the 23S rRNA gene were involved in clarithromycin resistance, an 850-bp fragment of the 23S rRNA gene from 17 clinical isolates was amplified and the nucleotide sequences were determined. There were a total of 11 sensitive and six resistant isolates. All clarithromycin-resistant isolates contained an A→G transition mutation at nucleotide position 2515, while all of the clarithromycin-sensitive strains showed the wild-type sequence at this position.

The 23S rRNA gene fragments amplified by primers 23SR and 23SL from NTUH-CR1 were introduced into clarithromycin-sensitive *H. pylori* by natural transformation. Resistant *H. pylori* mutant strains were selected

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   1 GATCAATGAT GGCGCGAGTA TTATCATTTT ATGCAGCGCT CAAAAAGCGC AAAAATTGGG
  61 GTTAAAAGCA ATGGCAACTA TCAAGGGGTT TGGTTTGGGT GGTTGCAGTC CGGATATAAT
 121 GGGGATATGC CCTAGTATTG CTATTAAAAA CAATCTTAAA AATGTCAAAA TGAATCTCAA
 181 GGACATCAAT CTTTTTGAAC TCAATGAAGC CTTCGCCGCA CAAAGCATAG CCGTGTTAAA
 241 AGAGCTTGAA TTAAACCCCA ATATCGTGAA TGTGAATGGA GGCGCGATAA CCATTGGCCA
 301 CCCTATTGGT GCAAGCGGCG CTAAGATTTT GGTAACCTTA TTGCATGAAA TGAAAAGAAG
 361 CGCTCATGGC GTGGGCTGCG CGTCATTGTG TGTGGGCGGC GGACAAGGGC TATCAGTAAT
 421 AGTGGAACAA AAATAAGGAG AATGAGATGA ACAAGGTTAT AACCGATTTA GACAAAGCAT
                     RBS        M N   K V I   T D L   D K A L
 481 TGAGCGGGTT AAAAGACGGG GACACTATTT TAGTGGGCGG TTTTGGGCTG TGCGGGATAC
       S G L   K D G   D T I L   V G G   F G L   C G I P
 541 CCGAATACGC CATTGACTAC ATTTACAAGA AAGGCATCAA GGATTTGATT GTTGTGAGCA
       E Y A   I D Y   I Y K K   G I K   D L I   V V S N
 601 ATAATTGTGG CGTTGATGAC TTTGGGCTTG GTATTCTTTT AGAAAAAAAG CAGATTAAAA
       N C G   V D D   F G L G   I L L   E K K   Q I K K
 661 AGATTATCGC TTCCTATGTG GGGGAGAATA AGATTTTTGA ATCACAAATG CTGAACGGAG
       I I A   S Y V   G E N K   I F E   S Q M   L N G E
 721 AAATTGAAGT CGTTTTGACA CCGCAAGGCA CGCTGGCTGA AAACTTGCGC GCTGGTGGGG
       I E V   V L T   P Q G T   L A E   N L R   A G G A
 781 CTGGGATACC CGCTTACTAC ACCCCAACCG GTGTTGGGAC TTTGATCGCT CAAGGCAAAG
       G I P   A Y Y   T P T G   V G T   L I A   Q G K E
 841 AATCAAGGGA ATTTAACGGC AAAGAGTATA TTTTAGAAAG AGCGATAACA GGCGATTACG
       S R E   F N G   K E Y I   L E R   A I T   G D Y G
 901 GGCTTATCAA AGCCTATAAA AGCGATACTC TTGGGAACTT GGTGTTTAGA AAAACGGCTA
       L I K   A Y K   S D T L   G N L   V F R   K T A R
 961 GAAACTTTAA TCCCTTGTGC GCGATGGCGG CAAAAATATG CGTCGCTGAA GTGGAAGAAA
       N F N   P L C   A M A A   K I C   V A E   V E E I
1021 TTGTCCCGGC CGGGGAATTG GACCCAGATG AAATACACTT GCCAGGAATC TATGTGCAAC
       V P A   G E L   D P D E   I H L   P G I   Y V Q H
1081 ACATCTATAA GGGTGAGAAA TTTGAAAAAC GGATAGAAAA AATCACAACA AGGAGCGCAA
       I Y K   G E K   F E K R   I E K   I T T   R S A K
                                                     RBS
1141 AATGAGAGAG GCTATCATTA AAAGAGCGGC AAAGGAATTG AAAGAGGGCA TGTATGTGAA
      M R E   A I I K   R A A   K E L   K E G   M  Y V N
1201 TTTAGGGATA GGCTTGCCCA CGCTTGTGGC TAATGAAGTG AGCGGGATGA ATATCGTTTT
       L G I   G L P   T L V   A N E V   S G M   N I V F
1261 CCAAAGCGAG AACGGGTTAT TAGGGATTGG CGCTTACCCT TTAGAGGGGG GCGTTGATGC
       Q S E   N G L   L G I G   A Y P   L E G   G V D A
1321 GGATC
      D
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Fig. 3. *Full length sequence of clone 6. The coding region of* yxjD *starts at nucleotide 447.*

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HP0691  MNKVITDLDKALSALKDGDTILVGGFGLCGIPEYAIDYIYKKGIKDL
HPYxjD  --------N----G--------L------------------------
BSYxjD  -G--LSSSKE-AKLIH----LIA----------QL-LS-RDQ-V---

HP0691  IVVSNNCGVDDFGLGILLEKKQIKKIIASYVGENKIFESQMLNGEIE
HPYxjD  -----------------------------------------------
BSYxjD  T----------W-L-L--AN-----M------------R-F-S--L-

HP0691  VVLTPQGTLAENLHAGGAGIPAYYTPTGVGTLIAQGKESREFNGKEY
HPYxjD  -------------R---------------------------------
BSYxjD  -E-V-------RIR-------GF--A-----S--E---HKT-G-RT-

HP0691  ILERAITGDYGLIKAYKSDTLGNLVFRKTARNFNPLCAMAAKICVAE
HPYxjD  -----------------------------------------------
BSYxjD  V---G----VAIV--W-A--M---I----------IA---G--TI--

HP0691  VEEIVPAGELDPDEIHLPGIYVQHIYKGEKFEKRIEKITTRSTK
HPYxjD  ------------N-----------------------------A-
BSYxjD  A----E-------H--T-------VVL-ASQ------R-VQ---
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Fig. 4. *Alignment of the deduced amino acid sequences of the CoA-transferase subunits from* Helicobacter pylori *strain 26695 (HP0691) [37], the Taiwanese* H. pylori *isolate (HPYxjD) [this study], and* Bacteroides subtilis *(BSYxjD) [Genbank accession number Z99124].*

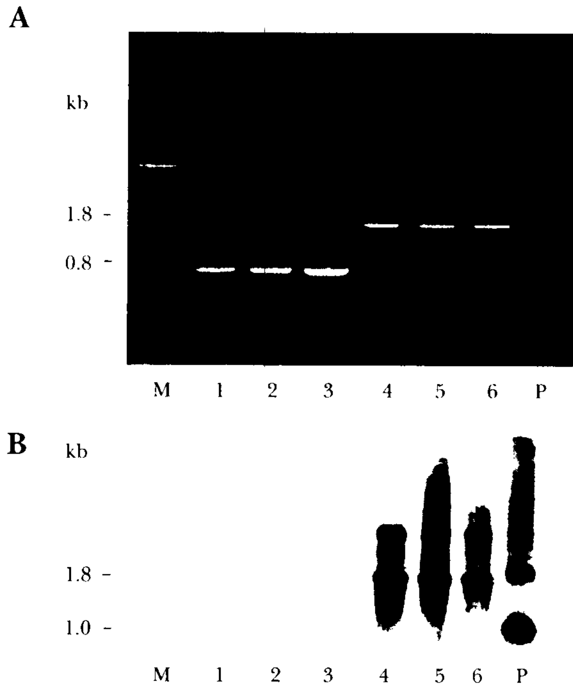

Fig. 5. *A) Gel electrophoresis and B) Southern blot analysis of PCR products amplified from* yxjD *wild-type and mutant genomic DNA of* Helicobacter pylori *strains. Lanes 1–3: wild-type* yxjD *gene. Lanes 4–6:* yxjD *gene knockout mutant. Lane M: molecular size markers. The chloramphenical acetyltransferase cassette was used as the probe for Southern blot analysis and as a positive control (Lane P).*

on plates with various concentrations of clarithromycin. Strains transformed by the 23S rRNA gene had greater than 1,000 colonies while none of the control group were able to grow at clarithromycin concentrations of at least 0.1 μg/mL. MICs of clarithromycin increased from less than 0.1 μg/mL to at least 15 to 20 μg/mL (~ 1,000-fold) in the transformants (Table 4).

Table 3. Minimum inhibitory concentrations of clarithromycin in wild-type and *yxjD* knockout mutant *Helicobacter pylori* strains

H. pylori strain	Methods	
	E-test (μg/mL)	Agar dilution test (μg/mL)
NTUH-CR2: wild type	12	15–20
NTUH-CR2: mutant*	8–12	15–20
NTUH-CS1: wild type	0.03	0.05
NTUH-CS1: mutant*	0.047	0.05
UA802: wild type	0.023	0.05–0.1
UA802: mutant*	0.032	0.0

*yxjD knocked out by CAT cassette.

Table 4. Minimum inhibitory concentrations of clarithromycin in wild-type *Helicobacter pylori* and strains with the A2515G 23S rRNA gene mutation

H. pylori strain	Methods	
	E-test (μg/mL)	Agar dilution test (μg/mL)
NTUH-CS1: wild type	0.032	0.01–0.05
NTUH-CS1: A2515G*	16–24	15–20
UA802: wild type	0.023	0.05–0.1
UA802: A2515G*	16	15

*23S rRNA A to G mutation induced by transformation of PCR product from NTUH-CR1 and confirmed by direct sequencing.

Sequencing of the clarithromycin-resistant transformants revealed an A→G mutation at position 2515 of the 23S rRNA gene in all cases. Degenerate PCR yielded products of various size under various conditions. However, sequencing of these PCR products failed to detect genes homologous to any genes reported to be associated with macrolide resistance (data not shown).

Discussion

Clarithromycin is a newly adopted antibiotic in the eradication therapy of *H. pylori* [36]. However, development of clarithromycin resistance during therapy can result in treatment failure. We therefore explored possible mechanisms of clarithromycin resistance in *H. pylori* by using various approaches, including degenerate PCR, and screening of an expression library in *E. coli*. Sequences of the 23S rRNA gene of *H. pylori* strains were determined to confirm the previously reported mechanisms.

Our study using degenerate PCR failed to identify genes homologous to other macrolide resistance-associated genes. Full genome sequencing also confirmed that there are no such homologues in the *H. pylori* genome [34]. Therefore, these mechanisms are unlikely to exist in *H. pylori*.

Screening of *E. coli* transformed with an expression library from *H. pylori* NTUH-CR1 resulted in isolation of six clarithromycin-resistant transformants, four of which contained the *H. pylori* 3-oxoadipate CoA-transferase gene. However, results of the knockout mutant and antibiotic inactivation assays suggested that the increased clarithromycin resistance was probably an effect of over-expression in *E. coli*, and that this gene is not involved in clarithromycin resistance in *H. pylori*. PCR products of the genomic DNA from the mutants showed only a single band. The complete disappearance of the wild-type gene products confirmed that the gene had been knocked out genetically. Although we did not perform additional biochemical assays, the residual RNA at the 5' end of the CAT cassette should be very unstable and rarely functional [31]. The knockout experiment also showed that this gene was not essential for clarithromycin resistance in *H. pylori*.

A→G and A→C mutations in both nucleotides 2514 and 2515 of the 23S rRNA gene have been observed in Western countries [24, 25, 37]. When MICs to clarithromycin were determined, strains with mutations at position 2514 seemed to have higher MICs than those with mutations at 2515. However, only the A→G mutation at position 2515 was detected in our Taiwanese strains. There are two copies of the 23S rRNA gene in the *H. pylori* genome [37, 38]. Because there was only one major peak found by PCR and direct sequencing, both copies of the 23S rRNA genes were mutated. This is in agreement with a previous report [37]. The reason that all six resistant strains had mutations at the same position (A2515G) is unknown. Further study is needed for clarification.

The transcription start site of 23S rRNA in *H. pylori* has recently been determined by primer extension assay [37]. From these results, nucleotide 2515 should be renamed as 2143. However, we use the old position for easy comparison with previous reports. We also demonstrated that this mutation was sufficient for clarithromycin resistance by natural transformation and mutagenesis in sensitive strains. The results of natural transformation also suggest the possibility of horizontal transfer of the 23S rRNA gene mutation and spread of drug resistance.

Macrolides such as erythromycin and clarithromycin inhibit nascent peptide chain elongation by interacting with the 50S ribosomal subunit and stimulating the release of peptidyl-tRNA from the A site [38, 39]. Footprinting experiments have demonstrated a direct physical interaction between erythromycin and the conserved region V of *E. coli* 23S rRNA. Specifically, the 23S rRNA residues A2058 and 2059 were protected when intact 70S ribosomes were incubated directly with erythromycin. Mutations conferring macrolide resistance occurred at or adjacent to the site of methylation. A→G mutations in 23S rRNA may cause macrolide resistance in *H. pylori* by the same mechanism

We conclude that mutation in the 23S rRNA gene is sufficient to confer clarithromycin resistance and that it is the major mechanism of clarithromycin resistance in *H. pylori*. All clarithromycin-resistant *H. pylori* strains in our study contained an A→G mutation at nucleotide position 2515 of the 23S rRNA gene. Over-

expression of 3-oxoadipate CoA-transferase elevates clarithromycin resistance in *E. coli*, but does not seem to have a significant role in clarithromycin resistance in *H. pylori*.

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