Discovery of novel mutations for clarithromycin resistance in Helicobacter pylori by using next-generation sequencing

Tran Thanh Binh1,2, Seiji Shiota1, Rumiko Suzuki1, Miyuki Matsuda1, Tran Thi Huyen Trang1, Dong Hyeon Kwon3, Shun Iwatani1,4 and Yoshio Yamaoka1,4*

1Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Oita, Japan; 2Department of Endoscopy, Cho Ray Hospital, Ho Chi Minh, Vietnam; 3Biology Department, Long Island University, Brooklyn, NY, USA; 4Department of Medicine-Gastroenterology, Baylor College of Medicine and Michael E. DeBakey Veterans Affairs Medical Center, Houston, TX, USA

*Corresponding author. Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, 1-1 Idaigaoka, Hasama-machi, Yufu-City, Oita 879-5593, Japan. Tel: +81-97-586-5740; Fax: +81-97-586-5749; E-mail: yyamaoka@oita-u.ac.jp

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Objectives: Resistance to clarithromycin is the most important factor causing failure of Helicobacter pylori eradication. Although clarithromycin resistance is mainly associated with three point mutations in the 23S rRNA genes, it is unclear whether other mutations are associated with this resistance.

Methods: Two types of clarithromycin-resistant strains (low- and high-resistance strains) were obtained from clarithromycin-susceptible H. pylori following exposure to low clarithromycin concentrations. The genome sequences were determined with a next-generation sequencer. Natural transformation was used to introduce the candidate mutations into strain 26695. Etest and an agar dilution method were used to determine the MICs.

Results: High-resistance strains contained the mutation A2143G in the 23S rRNA genes, whereas low-resistance strains did not. There were seven candidate mutations in six genes outside of the 23S rRNA genes. The mutated sequences in hp1048 (infB), hp1314 (rpl22) and the 23S rRNA gene were successfully transformed into strain 26695 and the transformants showed an increased MIC of and low resistance to clarithromycin. The transformants containing a single mutation in infB or rpl22 (either a 9 bp insertion or a 3 bp deletion) or the 23S rRNA gene showed low MICs (0.5, 2.0, 4.0 and 32 mg/L, respectively) while the transformants containing double mutations (mutation in the 23S rRNA genes and mutation in infB or rpl22) showed higher MICs (>256 mg/L).

Conclusions: Next-generation sequencing can be a useful tool for screening mutations related to drug resistance. We discovered novel mutations related to clarithromycin resistance in H. pylori (infB and rpl22), which have synergic effects with 23S rRNA resulting in higher MICs.

Keywords: 23S rRNA, rpl22, infB, in vitro, natural transformation

Introduction

Helicobacter pylori is a spiral-shaped, Gram-negative bacterium that is responsible for infections affecting more than half of the world’s population and is currently known to play a causative role in the pathogenesis of chronic gastritis, peptic ulcer diseases, gastric cancer and mucosa-associated lymphoid tissue lymphoma.1,2 Eradication of H. pylori not only improves peptic ulcer healing but also prevents its recurrence and reduces the risk of developing gastric cancer.3,4 Furthermore, other H. pylori-related disorders, such as mucosa-associated lymphoid tissue lymphoma, atrophic gastritis and intestinal metaplasia, have been shown to regress after antibiotic treatment.5,6 Triple therapy regimens including one proton pump inhibitor and two antimicrobial agents, such as amoxicillin and clarithromycin, have been widely used to eradicate this bacterium.5,8-10 Treatment success depends on several factors, such as smoking, patient compliance and antibiotic resistance.11-13 Resistance to clarithromycin is the most important factor in treatment failure.11,12,14 The prevalence of antibiotic resistance in H. pylori is now increasing worldwide and becoming a growing public health problem that needs more attention.5,12-17 More than 90% of clarithromycin-resistant strains have up to three point mutations in the peptidyltransferase region of domain V of 23S ribosomal RNA (rRNA): substitutions from adenine to guanine at position 2143 (A2143G) and those from adenine to...
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Antibiotic susceptibility testing

Etest (AB Biodisk, Solna, Sweden) and an agar dilution method were used to determine the MICs of clarithromycin. Briefly, Mueller–Hinton II agar medium supplemented with 10% defibrinated horse blood was used as culture medium and the culture suspension turbidity, which was adjusted to be equivalent to that of a McFarland opacity standard of 1.0–2.0, was used to inoculate the plates. The clarithromycin Etest strip was placed on the plate and incubated for 3–5 days at 37°C under microaerophilic conditions. Agar dilution MIC tests were performed according to the standard method recommended by CLSI.19 The plates contained 2-fold dilutions of clarithromycin with concentrations ranging from 0.0039 to 256 mg/L. MIC values were defined by the point of intersection of the inhibition ellipse zone with the graded strip for the Etest and the lowest concentration of clarithromycin that completely inhibited visible growth for the agar dilution method. Strains were considered resistant when the MIC value of clarithromycin was ≥ 1 mg/L.19,31 To confirm the results, we performed MIC tests at least three times using both the Etest and the agar dilution method at different times.

Determination of candidate mutations

We sequenced the genome DNA of three in vitro H. pylori strains: 26695-1 and two clarithromycin-resistant strains obtained by in vitro selection using a next-generation sequencer (90 bp pair-end, library length 500 bp, HiSeq2000; Illumina, Inc., San Diego, CA, USA). The whole genome sequences of three strains were reconstructed by mapping the short read sequences (90 bp pair-end) on the genome sequences of strain 26695 using CLC Genomics Workbench v4.0 (CLC bio, Aarhus, Denmark). In addition to reconstructing genomes by reference mapping, we also assembled de novo contigs using the same software. Candidate mutations were obtained by comparing the reconstructed genomes of the resistant strains with that of the wild-type strain 26695-1. To avoid misreading of the next-generation sequencer, we confirmed candidate mutations with PCR-based sequencing. The PCR conditions were as follows: initial denaturation for 5 min at 94°C, 35 amplification steps (94°C for 30 s, 55°C for 30 s and 72°C for 30 s) and a final extension cycle of 7 min at 72°C using Blend Taq DNA polymerase (Toyobo, Otsu, Japan). Amplified PCR products were purified using a QIAquick purification kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions and the amplified fragments were sequenced with a Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 3130 genetic analyser (Applied Biosystems) according to the manufacturer’s instructions. The sequences of candidate genes were then aligned with the reference sequences of those of strain 26695 deposited in GenBank using MEGA 5.0 free software (Molecular Evolutionary Genetics Analysis, Tempe, AZ, USA).

Natural transformation of the candidate mutations

Amplified PCR products containing candidate mutations and no mutations were obtained via PCR (Table S1, available as Supplementary data at JAC Online) and were separately introduced into clarithromycin-susceptible H. pylori 26695 through natural transformation by established methods as previously described.35–37 Briefly, recipient cells were inoculated onto Mueller–Hinton II agar plates and were grown for 5 h. Then, 0.5 μg of DNA (PCR fragments) diluted in TE (10 mM Tris–HCL, pH 8.0/1 mM EDTA) was added to the bacterial lawn. After incubation for 24 h under microaerophilic conditions, the transformed cells were spread onto Mueller–Hinton II agar plates containing clarithromycin (0.0039, 0.0078, 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0 mg/L) and several single colonies were separately collected from the lowest to the highest concentrations of clarithromycin-containing plates where they were seen and spread onto clarithromycin-free horse agar plates.
blood agar plates. The bacteria from each single colony were harvested and diluted in Brucella broth after incubation for 3–4 days. The culture medium was further inoculated onto clarithromycin-free horse blood agar plates at least three times before being used for evaluation of clarithromycin susceptibility with Etest and agar dilution methods. Successful transformations and mutations were confirmed with PCR followed by DNA sequencing analysis. For double mutation induction, amplified PCR products containing A2143G in the 23S rRNA genes were introduced into the successful transformants and amplified PCR fragments containing successful transformed candidate mutations were then introduced into each other. In the experiments, amplified PCR products containing A2143G in 23S rRNA were used as a positive control. Amplified PCR products containing no mutations in 23S rRNA and the parental strain 26695 were used as a negative control. Each natural transformation was performed at least twice at different times.

Results

Establishment of resistant strains

Wild-type strain 26695 (here denoted 26695-1), which is susceptible to clarithromycin, was exposed to low concentrations of clarithromycin in vitro and two clarithromycin-resistant strains were obtained. One strain, denoted 26695-1CL, was obtained through exposure to clarithromycin on lower concentration plates (up to 0.125 mg/L) and another strain, denoted 26695-1CH, was obtained through exposure to clarithromycin on higher concentration plates (up to 0.5 mg/L). The final MICs of clarithromycin for the two strains were 4 mg/L for strain 26695-1CL and >256 mg/L for strain 26695-1CH. The MIC of clarithromycin for strain 26695-1 was 0.03 mg/L.

Detection of mutations in resistant strains using next-generation sequencing

We sequenced the genome DNA of the in vitro H. pylori strains 26695-1, 26695-1CL and 26695-1CH with a next-generation sequencer. We mapped the short read sequences of H. pylori 26695-1, 26695-1CL and 26695-1CH to the 26695 genome with coverage depths of 733, 1233 and 824, respectively. We compared the resulting genome sequences of the three strains with the genome sequence of 26695 and detected 39 non-identical loci. We also performed de novo assembly for H. pylori 26695-1, 26695-1CL and 26695-1CH and constructed 42 contigs (in total 1629941 bp), 45 contigs (1628730 bp) and 43 contigs (1631403 bp), respectively. By comparison of the de novo contigs, we detected one polymorphic locus in a repeated region.

Finally, we identified 40 polymorphic loci [19 single-nucleotide polymorphisms (SNPs) and 21 indels; data not shown] between the three strains and strain 26695. Of these 40 loci, mutations in 13 loci (nine SNPs and four indels) were shared among the three strains and regarded as strain-specific variants that existed before the acquisition of drug resistance. Mutations in six loci (two SNPs and four indels) were observed in the wild-type strain but were uncommon in the clarithromycin-resistant strains or the original strain 26695. These loci (19 total) were excluded from the subsequent analysis. Finally, we obtained a list of 21 mutations (8 SNPs and 13 indels) in 14 genes as candidate mutations occurring in strain 26695-1CL, 26695-1CH or both (Table S2, available as Supplementary data at JAC Online). Six mutations in six genes were found in both 26695-1CL and 26695-1CH. Eight mutations in seven genes were found only in strain 26695-1CL. Furthermore, seven mutations in six genes were found only in strain 26695-1CH.

Candidate mutations were confirmed using PCR and sequencing. A total of nine mutations (seven SNPs, one insertion and one deletion) in eight genes were confirmed as candidate mutations (Table 1). Gene hp0190, which encodes a conserved hypothetical secreted protein whose function is unknown, contained a mutation in both 26695-1CL and 26695-1CH. These two strains also contained mutations in hp0471 (kefB, a glutathione-regulated potassium-efflux system protein) and hp1048 (infB, translation initiation factor IF-2, which protects formylmethionyl-transfer RNA from spontaneous hydrolysis, promotes its binding to 30S ribosomal subunits during the initiation of protein synthesis and is involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex). One point mutation (C2398A) in hp0607 (acrB, an acriflavine resistance protein) and a 9 bp insertion in hp1314 (rpl22, ribosomal protein L22) were found only in strain 26695-1CL. Interestingly, rpl22 encodes a core protein of the large ribosomal subunit interacting with all domains of 23S rRNA related to erythromycin resistance in Escherichia coli. On the other hand, one point mutation (A571G) in hp1171, which encodes a glutamine ABC transporter ATP-binding protein, was found in strain 26695-1CH. A 3 bp deletion in rpl22 was also found only in strain 26695-1CH. Of note, genes rrnA23S and rrnB23S, which are the two copies of the 23S rRNA genes associated with macrolide resistance, contained the mutation A2143G, equivalent to the mutation A2143G in domain V of the 23S rRNA genes as proposed by Taylor et al.38 Interestingly, strain 26695-1CH contained this mutation, whereas no point mutation in the 23S rRNA genes was found in strain 26695-1CL. These data suggested that point mutation in acrB mutation (9 bp insertion) in rpl22 or both are involved in clarithromycin resistance independent of point mutation in 23S rRNA.

Confirmation that the mutations are involved in resistance using natural transformation

To determine whether the seven mutations in six genes (hp0190, kefB, acrB, infB, hp1171 and rpl22) outside the 23S rRNA genes were involved in clarithromycin resistance, we performed natural transformation of the mutated PCR products into the clarithromycin-susceptible H. pylori strain 26695 using the established method. Transformed cells were selected on Mueller–Hinton II agar plates supplemented with serial concentrations of clarithromycin using an agar dilution method (from 0.0039 to 256 mg/L by doubling dilution). Seven candidate mutations were separately introduced into wild-type strain 26695 (Table 2). However, we could not obtain any transformants for four candidate mutations in the genes hp0190, kefB, acrB and hp1171 even on the plates containing 0.03 mg/L clarithromycin. In contrast, colonies from each candidate mutation in infB (named [infB, G160A]), a 9 bp insertion and a 3 bp deletion in rpl22 (named [rpl22, 9 bp insertion]) and [rpl22, 3 bp deletion], respectively) were successfully obtained from plates containing clarithromycin at 0.03–0.06, 0.03–0.05 and 0.03–0.125 mg/L, respectively (Table 2). At least eight colonies from the lowest to the highest clarithromycin concentration plates were obtained for further evaluation of MICs and the final MICs were 0.5 mg/L for [infB, G160A], 2.0 mg/L for [rpl22, 9 bp insertion] and 4.0 mg/L for
Novel mutations for clarithromycin resistance in *H. pylori*

**Table 1.** Nine mutations in eight genes confirmed with PCR and sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Position of mutations</th>
<th>Mutation type</th>
<th>No.</th>
<th>Wild-type</th>
<th>Mutation</th>
<th>26695-1CL</th>
<th>26695-1CH</th>
<th>26695-1f</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrB (hp0607)</td>
<td>2398</td>
<td>SNP</td>
<td>1</td>
<td>C</td>
<td>A</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>infB (hp1048)</td>
<td>160</td>
<td>SNP</td>
<td>1</td>
<td>G</td>
<td>A</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>kefB (hp0471)</td>
<td>743</td>
<td>SNP</td>
<td>1</td>
<td>T</td>
<td>A</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>rpl22 (hp1314)</td>
<td>295</td>
<td>indel</td>
<td>9</td>
<td>TTTTATTTT</td>
<td>insertion</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>rpl22 (hp1314)</td>
<td>226</td>
<td>indel</td>
<td>3</td>
<td>GTG</td>
<td>deletion</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>hp0190</td>
<td>712</td>
<td>SNP</td>
<td>1</td>
<td>C</td>
<td>T</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>hp1171</td>
<td>571</td>
<td>SNP</td>
<td>1</td>
<td>A</td>
<td>G</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>rrnA23S</td>
<td>2147</td>
<td>SNP</td>
<td>1</td>
<td>A</td>
<td>G</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>rrnB23S</td>
<td>2147</td>
<td>SNP</td>
<td>1</td>
<td>A</td>
<td>G</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

acrB, gene encoding acriflavine resistance protein; infB, gene encoding translation initiation factor IF-2; kefB, gene encoding glutathione-regulated potassium-efflux system protein; rpl22, gene encoding core protein of the large ribosomal subunit.

+, a nucleotide mutation or insertion or deletion mutations occurred.

–, no nucleotide mutations or insertion or deletion mutations occurred.

aNumber of nucleotides in SNP or insertion or deletion mutations.

bNucleotide or nucleotide segment in wild-type strain.

cNucleotide mutation or insertion or deletion mutations found in mutant strains.

dA low-MIC clarithromycin-resistant strain.

eA high-MIC clarithromycin-resistant strain.

fWild-type strain did not contain this segment.

**Table 2.** Seven candidate mutations introduced into strain 26695 via natural transformation using an agar dilution method under clarithromycin selection

<table>
<thead>
<tr>
<th>PCR products for genes containing mutations</th>
<th>Maximum clarithromycin concentration (mg/L) at which transformants were recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrB, C2398A</td>
<td>0.015</td>
</tr>
<tr>
<td>infB, G160A</td>
<td>0.06</td>
</tr>
<tr>
<td>kefB, T743A</td>
<td>0.015</td>
</tr>
<tr>
<td>rpl22, 9 bp insertion</td>
<td>0.5</td>
</tr>
<tr>
<td>rpl22, 3 bp deletion</td>
<td>0.125</td>
</tr>
<tr>
<td>hp0190, C712T</td>
<td>0.015</td>
</tr>
<tr>
<td>hp1171, A571G</td>
<td>0.015</td>
</tr>
<tr>
<td>23S, A2143G (positive control)</td>
<td>8</td>
</tr>
<tr>
<td>23S, A2143A (negative control)</td>
<td>0.015</td>
</tr>
<tr>
<td>26695 (negative control)</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Bold formatting shows that the transformant showed resistance to clarithromycin.

aPCR products containing mutation A2143G in 23S rRNA were introduced into wild-type strain 26695; used as a positive control.

bPCR products without any mutations in 23S rRNA were introduced into wild-type strain 26695; used as a negative control.

cWild-type strain 26695 was also used as a negative control.

[rpl22, 3 bp deletion] (Table 3). The corresponding mutations in each transformant were confirmed with PCR-based sequencing. We also confirmed that no mutations (A2143G, A2142G and A2142C) were present in the 23S rRNA genes of all these selected transformants. PCR fragments containing a mutation at A2143G in the 23S rRNA genes obtained from strain 26695-1CH were used as positive control PCR products. Several colonies (named [26695 + 23S, A2143G]) were observed on the plates beginning with clarithromycin concentrations from 0.03 to 8.0 mg/L (Table 2). At least eight colonies from these clarithromycin concentration plates were observed only in the plates containing 0.015 mg/L clarithromycin. The final MICs for all selected [26695 + 23S, A2143G] colonies were 32 mg/L (Table 3). The mutation A2143G in the 23S rRNA genes was confirmed from all positive control transformants with PCR-based sequencing. None of seven candidate mutations in these transformants was found. To avoid concern about spontaneous mutation, we also transformed clarithromycin-susceptible strain 26695 with the PCR products that were amplified with the same primers as those used for the 23S rRNA genes but without any mutations as a negative control. We did not observe any colonies in repeated experiments even with plates containing 0.03 mg/L clarithromycin (colonies were observed only on plates containing 0.015 mg/L clarithromycin). We also used clarithromycin-susceptible strain 26695 without transformation and showed that the colonies were obtained only on plates containing 0.015 mg/L clarithromycin.

To evaluate the role of candidate mutations in combination effects, we performed further natural transformations with the same method as that used for single transformation. PCR fragments containing A2143G obtained from strain 26695-1CH were introduced into the [infB, G160A], [rpl22, 9 bp insertion] and [rpl22, 3 bp deletion] transformants (named [infB, G160A + A2143G], [rpl22, 9 bp insertion + A2143G] and [rpl22, 3 bp deletion + A2143G], respectively) and we obtained transformants containing double mutations in the 23S rRNA genes and either infB or rpl22 (9 bp insertion or 3 bp deletion), confirmed with PCR-based sequencing. These transformants showed much higher MICs (>256 mg/L) than those for transformants with any single mutation (Table 3). PCR fragments containing a mutation in rpl22 (9 bp insertion) obtained from [rpl22, 9 bp insertion]...
transformants were also introduced into the [infB, G160A] trans- 
formants (named [infB, G160A + rpl22, 9 bp ]). Transforms containing double mutations in infB and rpl22 (9 bp insertion) 
showed slightly higher MICs than those for transformants 
containing single mutations (4.0 versus 0.5 and 2.0 mg/L, 
respectively).

Discussion

Clarithromycin is a key antimicrobial agent in standard triple 
therapy regimens for the eradication of H. pylori infection. Resistance 
to clarithromycin in H. pylori is becoming common and is the 
most important factor in treatment failure. Most clarithromycin-resistant strains have point mutations in the pep- 
tidyltransferase region of domain V of 23S rRNA; however, 
clarithromycin-resistant strains without mutation in 23S RNA 
also exist, indicating that unknown genes outside 23S rRNA are 
likely involved in clarithromycin resistance. In the present study, 
we successfully constructed two clarithromycin-resistant strains 
through exposure to low concentrations of clarithromycin and 
obtained novel candidate genes related to clarithromycin resist- 
ance using next-generation sequencing technology. Surprisingly,
sequences in only six genes outside the 23S RNA genes were 
confirmed to be mutated. We determined the significance of 
the novel mutations related to clarithromycin resistance through 
natural transformation experiments.

In control experiments using natural transformation, we con- 
firmed that transformants containing A2143G in 23S rRNA 
showed moderate resistance to clarithromycin (MIC of 32 mg/L). 
In addition, two mutations in rpl22 (either a 9 bp insertion or a 
3 bp deletion) and one mutation in infB were successfully trans- 
formed into wild-type strain 26695, and the transformants 
showed low resistance and increased MICs of clarithromycin 
(MICs of 2, 4 and 0.5 mg/L, respectively). We were also concerned 
that spontaneous mutations may occur under the selection by 
clarithromycin during the experiments, although the method- 
ology has been established and has been used not only for ex- 
periments with clarithromycin resistance but also with 
metronidazole, tetracycline and fluoroquinolones as well as 
amoxicillin. We transformed clarithromycin-susceptible 
strain 26695 with the PCR products that were amplified with the 
same primers as those used for the 23S RNA genes but without 
any mutations as a negative control, and did not obtain any col- 
onies on clarithromycin containing-plates (0.03 mg/L), suggesting 
the possibility that resistance caused by spontaneous mutations 
was rare.

rpl22 encodes ribosomal protein L22, a core protein of the large 
ribosomal subunit interacting with all domains of 23S RNA in 
which the triplet amino acid deletions at positions Met82-Lys83-
Arg84 in L22 causes resistance to erythromycin in E. coli. 
Erythromycin and clarithromycin, which are first- and second- 
generation macrolides, respectively, should have the same anti-
microbial activity and resistance mechanisms. In general, 
macrolide resistance is caused by several mechanisms, including 
target ribosomal modification, inactivation by enzymes, imper- 
meability of the bacterial membrane and active multidrug 
efflux. In the case of E. coli, the addition, deletion (or both) of one or more amino acids in ribosomal protein L22 can 
confer erythromycin resistance, for which the mechanisms 
related to L22 have been constructed and confirmed. Therefore, we assume that any mutations with deletion, insertion or 
both in rpl22 could be related to clarithromycin resistance. In 
fact, when we again constructed clarithromycin-resistant strains 
via exposure to low concentrations of clarithromycin, we obtained 
clarithromycin-resistant strains with another 3 bp deletion or 6 bp 
insertion in rpl22 without mutation in 23S RNA genes (T. T. Binh, 
Y. Yamaoka and S. Shiota, unpublished observation). In addition, 
we found a clarithromycin-resistant clinical isolate from 
Vietnam that had a 9 bp insertion in rpl22 (position 322; 
T. T. Binh, Y. Yamaoka and S. Shiota, unpublished observation). Overall, the novel mutations found in rpl22 clearly play an important 
role in clarithromycin resistance in H. pylori, especially in some 
clarithromycin-resistant strains without mutations in domain V of 
23S RNA. Furthermore, the presence/absence of the mutations in 
rpl22 can explain, at least in part, some controversial mutations 
in the 23S RNA genes that have been reported to confer 
clarithromycin resistance in the presence or absence of the efflux 
mechanism.

One mutation in infB was also transformed into wild-type 
strain 26695, and the transformants showed increased MICs of

Table 3. PCR-based sequencing results and final MICs for successful transformants

<table>
<thead>
<tr>
<th>Transformants with mutations</th>
<th>rpl22</th>
<th>infB</th>
<th>23S rRNA</th>
<th>Final MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[infB, G160A]</td>
<td>wild-type</td>
<td>G160A</td>
<td>wild-type</td>
<td>0.5</td>
</tr>
<tr>
<td>[rpl22, 9 bp insertion]</td>
<td>9 bp insertion</td>
<td>wild-type</td>
<td>wild-type</td>
<td>2</td>
</tr>
<tr>
<td>[rpl22, 3 bp deletion]</td>
<td>3 bp deletion</td>
<td>wild-type</td>
<td>wild-type</td>
<td>4</td>
</tr>
<tr>
<td>[26695 + 23S, A2143G]</td>
<td>wild-type</td>
<td>G160A</td>
<td>A2143G</td>
<td>32</td>
</tr>
<tr>
<td>[infB, G160A + 23S, A2143G]</td>
<td>wild-type</td>
<td>G160A</td>
<td>A2143G</td>
<td>&gt;256</td>
</tr>
<tr>
<td>[rpl22, 9 bp insertion + 23S, A2143G]</td>
<td>9 bp insertion</td>
<td>wild-type</td>
<td>A2143G</td>
<td>&gt;256</td>
</tr>
<tr>
<td>[rpl22, 3 bp deletion + 23S, A2143G]</td>
<td>3 bp deletion</td>
<td>wild-type</td>
<td>A2143G</td>
<td>&gt;256</td>
</tr>
<tr>
<td>[infB, G160A + rpl22, 9 bp insertion]</td>
<td>9 bp insertion</td>
<td>G160A</td>
<td>wild-type</td>
<td>4</td>
</tr>
<tr>
<td>Wild-type 26695</td>
<td>wild-type</td>
<td>wild-type</td>
<td>wild-type</td>
<td>0.03</td>
</tr>
</tbody>
</table>

G160A, substitution of adenine for guanine at position 160 in the infB gene.
A2143G, substitution of guanine for adenine at position 2143 in the 23S RNA genes.
Transformants carrying A2143G in the 23S RNA genes were used as a positive control.
Transformants containing 9 bp insertion in rpl22 were used as a negative control.
clarithromycin, although the final MIC (0.5 mg/L) did not reach the breakpoint of clarithromycin resistance. infB encodes translation initiation factor IF-2 (infB), which protects formylmethionyl-transfer RNA from spontaneous hydrolysis, promotes its binding to 30S ribosomal subunits during the initiation of protein synthesis and is involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex. In addition, infB was reported to specifically protect from chemical modification two sites in domain V of the 23S rRNA genes, strongly indicating that infB has a synergic effect in clarithromycin resistance mechanisms.95

Importantly, MICs for the transformants containing single mutations in infB and rpl22 (0.5–4 mg/L) increased when A2143G in 23S rRNA was introduced (>256 mg/L). The MIC for the transformants containing a single mutation in A2143G in 23S rRNA (32 mg/L) was lower than those for transformants with double mutations, clearly showing the existence of their synergic relationship with each other, although the effect of the A2143G mutation seemed to be stronger than that of mutations in rpl22 or infB. We also found that strain 26695-1CH carries mutations in the 23S rRNA, rpl22 and infB genes and showed a high MIC (>256 mg/L). Because the transformants containing A2143G in 23S rRNA and mutations in either rpl22 or infB showed a high MIC (>256 mg/L), all three mutations seem to be unnecessary, but mutations other than A2143G in 23S rRNA are clearly necessary for full resistance to clarithromycin. The clarithromycin-resistant strain 26695-1CL, with a lower MIC (4 mg/L), had mutations in both infB and rpl22 (9 bp insertion) but not in A2143G in 23S rRNA, and transformants containing a single mutation in infB or rpl22 (9 bp insertion) showed a lower MIC (0.5 and 2 mg/L, respectively) than that of 26695-1CL, which also indicated the presence of the synergic effect. Interestingly, when the transformants containing the two mutations in infB and rpl22 (9 bp insertion) were constructed, the MIC was similar to that for 26695-1CL (4 mg/L) (Table 3), confirming that the mutation in 23S rRNA is the major factor for clarithromycin resistance.

Our study has several limitations. We were unable to obtain transformants for four mutations in four genes (hp0190, kefB, acrB and hp1171). We do not know whether this was due to a lack of strong clarithromycin resistance phenotype when each candidate mutation was introduced into parental strain 26695 or whether some of the genes must be present together to cause co-effects in the clarithromycin resistance mechanism. However, we cannot deny the possibility that transformants containing mutations in only acrB, as well as in hp0190, kefB and hp1171, were not obtained via natural transformation under clarithromycin selection. We used simple PCR products with mutation for natural transformation, indicating the absence of selection with antibiotic cassettes (e.g. chloramphenicol selection). In particular, we believe that kefB, acrB and hp1171 are potential candidate genes related to clarithromycin resistance. Although kefB and hp1171 have not been previously reported in association with particular diseases or drug resistance, kefB encodes a glutathione-regulated potassium-efflux system protein and hp1171 encodes a glutamine ABC transporter ATP-binding protein, suggesting their putative roles in resistance, such as in an active efflux mechanism.94,95 Furthermore, some recent studies have reported that H. pylori contains an active multidrug efflux mechanism related to the development of resistance to clarithromycin, and acrB (hefC) was among the candidate gene clusters for the efflux pump in H. pylori, indicating the relevance of the gene to clarithromycin resistance mechanisms.18,57,58 We are now trying to transform these candidate mutations into 26695 via selection by using antibiotic cassettes to confirm whether they have roles in clarithromycin resistance. Further studies will be needed.

Finally, next-generation sequencing alone cannot read the whole genome, as one contig and some sequences of the genome may be read incompletely, especially in the repeated regions of the DNA sequences.25,59 Therefore, we may have missed some other mutations in some genes elsewhere that may be related to clarithromycin resistance. Nonetheless, we confirmed that next-generation sequencing technology is a useful tool for screening mutations related to drug resistance.

In conclusion, we analysed the genome profile for clarithromycin resistance in H. pylori using next-generation sequencing, showing that this new technology is useful in screening mutations related to drug resistance. Not only point mutations in the 23S rRNA genes but also novel mutations in rpl22 and infB were confirmed to be involved in increased resistance to clarithromycin.

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Transparency declarations
None to declare.

Supplementary data
Tables S1 and S2 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References