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

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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.[3–5] 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.[6,7] 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.[5,9,15–17]

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

guanine or cytosine at position 2142 (A2142G or A2142C).[17–21] Several other point mutations in the region (e.g. A2115G, T2117C, G2141A, A2144T, T2182C, G2223A, T2288C and T2711C) are also reportedly involved in clarithromycin resistance; however, the significance of these mutations remains controversial.[18,20,22,23] To date, it is unclear whether other mutations in genes outside domain V of 23S rRNA are associated with clarithromycin resistance.

In the more than three decades since the Sanger sequencing method was established, it has become the dominant and gold standard method for DNA sequencing. Although the technique has seen many improvements, its limitations, such as time and labour intensiveness and high cost, have highlighted the need for new and improved technologies for sequencing larger numbers of genomes. Next-generation sequencing technologies that can read enormous quantities of DNA sequences in less time and at lower cost than conventional sequencing have recently been introduced.[24,25] This new technology has been applied to clarify the evolution and pathogenicity of *H. pylori*, as well as to identify its novel virulence factors.[26–31] Another interesting practical application is the detection of genomic changes related to drug resistance through comparison of the genome of wild-type strains and those that survive antibiotic treatment; however, no reports to date have analysed the genome profile for clarithromycin resistance in *H. pylori* infection using next-generation sequencing. In this study, we clarified the mutations in genes outside domain V of 23S rRNA associated with clarithromycin resistance in *H. pylori* using this new technology.

Methods

In vitro selection of clarithromycin-resistant H. pylori strains

Wild-type strain 26695, here denoted 26695-1, which is susceptible to clarithromycin, was obtained from a subculture of original strain 26695 purchased from ATCC. Clarithromycin-resistant strains were constructed from strain 26995-1 following exposure to low concentrations of clarithromycin *in vitro* as described previously.[32,33] A single colony of 26995-1 was used to inoculate Mueller–Hinton II agar medium (Becton Dickinson, Sparks, MD, USA) supplemented with 10% defibrinated horse blood (Biotest Lab, Tokyo, Japan) without antibiotics. The plate was incubated at 37°C under microaerophilic conditions (10% O_2, 5% CO_2 and 85% N_2) for 72 h. Colonies on agar plates were harvested and put into Brucella Broth (Becton Dickinson, Sparks, MD, USA) containing 10% horse serum. Firstly, the culture medium was exposed to serially doubling concentrations of clarithromycin (0.0039, 0.0078, 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0 mg/L) via an agar dilution method. Then, colonies from the highest concentrations in which they could grow were obtained and repeatedly transferred to the same clarithromycin concentration five times before being exposed to the higher clarithromycin concentrations. The experiments were repeatedly performed until clarithromycin-resistant strains were obtained. Briefly, the cultures were allowed to reach a turbidity equivalent to that of a McFarland opacity standard of ∼1.0–2.0, and the culture medium containing bacteria was replicated directly onto the clarithromycin-containing dilution agar plates. The plates were incubated at 37°C under microaerophilic conditions. After being confirmed resistant to clarithromycin using an Etest and an agar dilution method, the isolates were also transferred to clarithromycin-free blood agar plates at least five times followed by a redetermination of final MICs to assess the stability of the selected resistance.

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.[34] 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 $\geq$1 mg/L.[19,34] 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, 2.0, 4.0, 8.0, 16, 32, 64, 128 and 256 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

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 A2147G, 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 independently 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, 0.03–0.5 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

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

Gene	Position of mutations	Mutation type	No.[a]	Wild-type[b]	Mutation[c]	26695-1CL[d]	26695-1CH[e]	26695-1[f]
acrB (*hp0607*)	2398	SNP	1	C	A	+	−	−
infB (*hp1048*)	160	SNP	1	G	A	+	+	−
kefB (*hp0471*)	743	SNP	1	T	A	+	+	−
rpl22 (*hp1314*)	295	indel	9	TTCCATGTA[g]	insertion	+	−	−
rpl22 (*hp1314*)	226	indel	3	GTG	deletion	−	+	−
hp0190	712	SNP	1	C	T	+	+	−
hp1171	571	SNP	1	A	G	−	+	−
rrnA23S	2147	SNP	1	A	G	−	+	−
rrnB23S	2147	SNP	1	A	G	−	+	−

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.
[a]Number of nucleotides in SNP or insertion or deletion mutations.
[b]Nucleotide or nucleotide segment in wild-type strain.
[c]Nucleotide mutation or insertion or deletion mutations found in mutant strains.
[d]A low-MIC clarithromycin-resistant strain.
[e]A high-MIC clarithromycin-resistant strain.
[f]A wild-type 26695 strain.
[g]Wild-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

PCR products for genes containing mutations	Maximum clarithromycin concentration (mg/L) at which transformants were recovered
acrB, C2398A	0.015
***infB*, G160A**	**0.06**
kefB, T743A	0.015
***rpl22*, 9 bp insertion**	**0.5**
***rpl22*, 3 bp deletion**	**0.125**
hp0190, C712T	0.015
hp1171, A571G	0.015
23S, A2143G (positive control)[a]	**8**
23S, A2143A (negative control)[b]	0.015
26695 (negative control)[c]	0.015

Bold formatting shows that the transformant showed resistance to clarithromycin.
[a]PCR products containing mutation A2143G in 23S rRNA were introduced into wild-type strain 26695; used as a positive control.
[b]PCR products without any mutations in 23S rRNA were introduced into wild-type strain 26695; used as a negative control.
[c]Wild-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 obtained for further evaluation of MICs and the confirmation of the mutation. 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]

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

Transformants with mutations	*rpl22*	*infB*	23S rRNA	Final MIC (mg/L)
[*infB*, G160A]	wild-type	G160A	wild-type	0.5
[*rpl22*, 9 bp insertion]	9 bp insertion	wild-type	wild-type	2
[*rpl22*, 3 bp deletion]	3 bp deletion	wild-type	wild-type	4
[26695+23S, A2143G][a]	wild-type	wild-type	A2143G	32
[*infB*, G160A+23S, A2143G]	wild-type	G160A	A2143G	>256
[*rpl22*, 9 bp insertion+23S, A2143G]	9 bp insertion	wild-type	A2143G	>256
[*rpl22*, 3 bp deletion+23S, A2143G]	3 bp deletion	wild-type	A2143G	>256
[*infB*, G160A+*rpl22*, 9 bp insertion]	9 bp insertion	G160A	wild-type	4
Wild-type 26695[b]	wild-type	wild-type	wild-type	0.03

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 rRNA genes.
[a]Transformants carrying A2143G in the 23S rRNA genes were used as a positive control.
[b]Wild-type strain 26695 was used as a negative control.

transformants were also introduced into the [*infB*, G160A] transformants (named [*infB*, G160A+*rpl22*, 9 bp]). Transformants 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.[11,12,14,15] Most clarithromycin-resistant strains have point mutations in the peptidyltransferase region of domain V of 23S rRNA; however, clarithromycin-resistant strains without mutation in 23S rRNA 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 resistance using next-generation sequencing technology. Surprisingly, sequences in only six genes outside the 23S rRNA 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 confirmed 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 transformed 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 methodology has been established and has been used not only for experiments with clarithromycin resistance[36,38–40] but also with metronidazole,[37] tetracycline[41] and fluoroquinolones,[42,43] as well as amoxicillin.[44,45] We 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, and did not obtain any colonies 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 rRNA in which the triplet amino acid deletions at positions Met82-Lys83-Arg84 in L22 causes resistance to erythromycin in *E. coli*.[46–49] Erythromycin and clarithromycin, which are first- and second-generation macrolides, respectively, should have the same antimicrobial activity and resistance mechanisms.[50,51] In general, macrolide resistance is caused by several mechanisms, including target ribosomal modification, inactivation by enzymes, impermeability of the bacterial membrane and active multidrug efflux.[49,52,53] 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.[47,48,54] 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 rRNA 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 rRNA. Furthermore, the presence/absence of the mutations in *rpl22* can explain, at least in part, some controversial mutations in the 23S rRNA genes that have been reported to confer clarithromycin resistance in the presence or absence of the efflux mechanism.[18]

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

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.[55]

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 lack of a 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.[49,56] 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/).

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