AAC Journals.ASM.org

Search for Novel Candidate Mutations for Metronidazole Resistance in *Helicobacter pylori* Using Next-Generation Sequencing

Tran Thanh Binh,[a,b] Rumiko Suzuki,[a] Tran Thi Huyen Trang,[a] Dong Hyeon Kwon,[c] Yoshio Yamaoka[a,d]

Department of Environmental and Preventive Medicine, Oita University Faculty of Medicine, Yufu, Japan[a]; Department of Endoscopy, Cho Ray Hospital, Ho Chi Minh City, Vietnam[b]; Biology Department, Long Island University, Brooklyn, New York, USA[c]; Department of Medicine-Gastroenterology, Baylor College of Medicine, and Michael E. DeBakey Veterans Affairs Medical Center, Houston, Texas, USA[d]

Metronidazole resistance is a key factor associated with *Helicobacter pylori* treatment failure. Although this resistance is mainly associated with mutations in the *rdxA* and *frxA* genes, the question of whether metronidazole resistance is caused by the inactivation of *frxA* alone is still debated. Furthermore, it is unclear whether there are other mutations involved in addition to the two genes that are associated with resistance. A metronidazole-resistant strain was cultured from the metronidazole-susceptible *H. pylori* strain 26695-1 by exposure to low concentrations of metronidazole. The genome sequences of both susceptible and resistant *H. pylori* strains were determined by Illumina next-generation sequencing, from which putative candidate resistance mutations were identified. Natural transformation was used to introduce PCR products containing candidate mutations into the susceptible parent strain 26695-1, and the metronidazole MIC was determined for each strain. Mutations in *frxA* (*hp0642*), *rdxA* (*hp0954*), and *rpsU* (*hp0562*) were confirmed by the Sanger method. The mutated sequence in *rdxA* was successfully transformed into strain 26695-1, and the transformants showed resistance to metronidazole. The transformants containing a single mutation in *rdxA* showed a low MIC (16 mg/liter), while those containing mutations in both *rdxA* and *frxA* showed a higher MIC (48 mg/liter). No transformants containing a single mutation in *frxA* or *rpsU* were obtained. Next-generation sequencing was used to identify mutations related to drug resistance. We confirmed that the mutations in *rdxA* are mainly associated with metronidazole resistance, and mutations in *frxA* are able to enhance *H. pylori* resistance only in the presence of *rdxA* mutations. Moreover, mutations in *rpsU* may play a role in metronidazole resistance.

Helicobacter pylori is a spiral-shaped Gram-negative bacterium that infects more than half of the world's population and is a major cause of chronic gastritis, peptic ulcer diseases, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (1, 2). The 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 antimicrobial therapy (6–8). Metronidazole has been used widely in combination therapies, such as metronidazole-based triple therapy, concomitant therapy, and bismuth-containing quadruple therapy, to eradicate this bacterium (5, 9, 10). Although treatment success depends on several factors, such as smoking status and patient compliance, antibiotic resistance is the major cause of treatment failure (11–13). However, along with clarithromycin resistance, resistance to metronidazole has arisen independently and is becoming increasingly common (14, 15).

Resistance to metronidazole was described previously and is predominantly associated with mutations in *rdxA* (*hp0954*), a gene encoding an oxygen-insensitive NAD(P)H nitroreductase (16–19). Mutations in two additional genes, *frxA* (*hp0642*) and *frxB* (*hp1508*, encoding a ferredoxin-like enzyme), both of which encode NAD(P)H-flavin oxidoreductases, have been shown to enhance *H. pylori* resistance when found along with *rdxA* gene mutations (20–23). However, the precise mechanism of metronidazole resistance is still debated, given that metronidazole resistance may also arise in *H. pylori* with mutations in the *frxA* gene only (22, 24). In addition, it is unclear whether other mutations in genes outside *rdxA* or *frxA* are associated with metronidazole resistance.

Recently, next-generation sequencing (NGS) has been applied to clarify the evolution and pathogenicity of *H. pylori*, as well as to identify its novel virulence factors (25–31). Another interesting practical application is the detection of genomic changes related to drug resistance through a comparison of the genomes of wild-type strains and of those that survive antibiotic treatment (32). Using next-generation sequencing, we are able to detect potential mutations throughout the genome of *H. pylori* and therefore identify novel mutations if they exist. In this study, we used next-generation sequencing to characterize the genomic changes associated with metronidazole resistance in *H. pylori*, where we confirmed mutations in the *frxA* and *rdxA* genes that are known to be associated with metronidazole resistance in *H. pylori*, and we identified a putative novel mutation in the *rpsU* gene that likely plays a role in metronidazole resistance.

Received 19 November 2014 **Returned for modification** 15 December 2014
Accepted 26 January 2015

Accepted manuscript posted online 2 February 2015

Citation Binh TT, Suzuki R, Trang TTH, Kwon DH, Yamaoka Y. 2015. Search for novel candidate mutations for metronidazole resistance in *Helicobacter pylori* using next-generation sequencing. Antimicrob Agents Chemother 59:2343–2348. doi:10.1128/AAC.04852-14.

Address correspondence to Yoshio Yamaoka yyamaoka@oita-u.ac.jp.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.04852-14.

doi:10.1128/AAC.04852-14

MATERIALS AND METHODS

***In vitro* selection of a metronidazole-resistant *H. pylori* strain.** The wild-type *H. pylori* strain 26695 (denoted 26695-1 in our previous study [32] and susceptible to metronidazole) was obtained from a subculture of the original 26695 strain purchased from the American Type Culture Collection (ATCC). A metronidazole-resistant strain was cultured from strain 26695-1 after exposure to low concentrations of metronidazole, as described previously (24, 32, 33). Briefly, a single colony of 26695-1 was inoculated onto Mueller-Hinton II agar (Becton Dickinson, Sparks, MD, USA) supplemented with 10% defibrinated horse blood (Nippon Biotest Lab, Tokyo, Japan) without antibiotics. The plate was incubated at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) for 72 h. The colonies were harvested and inoculated into brucella broth (Becton Dickinson) containing 10% horse serum. First, the culture medium was exposed to serially doubling concentrations of metronidazole (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 mg/liter) via an agar dilution method. The colonies exposed to the maximum concentrations of metronidazole were obtained and subcultured at the same concentration 5 times before being exposed to higher concentrations. Briefly, the cultures were allowed to reach a density of a McFarland opacity standard of approximately 1.0 to 2.0, and the culture medium containing bacterial cells was directly replicated onto the metronidazole-containing dilution agar plates. The plates were incubated at 37°C under microaerophilic conditions. After being confirmed resistant to metronidazole using an Epsilometer test (Etest) (AB Biodisk, Solna, Sweden) and agar dilution method, the isolates were transferred to metronidazole-free blood agar plates ≥5 times, followed by a redetermination of the final MICs to assess the stability of this resistance.

Antibiotic susceptibility testing. Etest and an agar dilution method were used to determine the MICs of metronidazole for the strain used in this study. Briefly, Mueller-Hinton II agar medium supplemented with 10% defibrinated horse blood was used as the culture medium, and the turbidity of the culture suspension, which was adjusted to be equivalent to a McFarland opacity standard of 1.0 to 2.0, was used for inoculation. The Etest strip of metronidazole was placed on the plate and incubated for 3 to 5 days at 37°C under microaerophilic conditions. The agar dilution MIC tests were performed according to the standard method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (34, 35). The plates contained 1.5-fold dilutions (to achieve small scales that agree with Etest) of metronidazole, with concentrations ranging from 1.0 to 128 mg/liter. The MIC was defined by the point of intersection of the inhibition ellipse zone with the graded strip for the Etest and the lowest concentration of metronidazole that completely inhibited visible growth for the agar dilution method. Strains were considered resistant when the MIC value was ≥8 mg/liter for metronidazole (34, 36). The MIC tests were performed for each putative resistant strain independently at least three times using both the Etest and agar dilution methods.

Determination of candidate mutations. *H. pylori* genomic DNA was extracted using the QIAamp DNA minikit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Genome resequencing was performed on the susceptible 26695-1 strain and one metronidazole-resistant strain obtained by *in vitro* selection using Illumina next-generation sequencing (HiSeq 2000; Illumina, Inc., San Diego, CA, USA). Raw sequencing reads (90-bp paired-end; mean insert size, 500 bp) were used to reconstruct the whole-genome sequences of both strains by mapping to the reference genome sequence of strain 26695 (GenBank accession no. NC_000915) using CLC Genomics Workbench version 4.0 (CLC bio, Aarhus, Denmark). Candidate mutations were obtained by comparing the reconstructed genomes of the two strains. The candidate mutations were confirmed by PCR using the primers listed in Table S1 in the supplemental material, followed by Sanger sequencing. The PCR in this study was carried out in 25-μl volumes containing 10× PCR buffer, 10 pmol of each primer, 2 mmol/liter $MgCl_2$, 200 μM each deoxyribonucleoside triphosphate (dNTP), 1 U of *Ex Taq* DNA polymerase (TaKaRa Bio, Inc., Otsu, Japan), and 20 to 50 ng of DNA. The PCR conditions were 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. The amplified PCR products were purified using the QIAquick purification kit (Qiagen, Inc.), according to the manufacturer's instructions, and the purified amplicons were sequenced with the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 3130 genetic analyzer (Applied Biosystems), according to the manufacturer's instructions. The sequences were then aligned using Molecular Evolutionary Genetics Analysis (MEGA) 6.0 (Tempe, AZ, USA) to the reference sequence of strain 26695 deposited in GenBank (accession no. NC_000915).

Natural transformation of the candidate mutations. The amplified PCR products containing either wild-type sequences or candidate mutations were separately introduced into metronidazole-susceptible *H. pylori* 26695-1 through natural transformation, as described previously (32, 37, 38). Briefly, recipient cells were inoculated onto Mueller-Hinton II agar plates and were grown for 5 h, after which 1.0 μg of PCR fragments diluted in TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) was added directly onto the bacterial lawn. After incubation for 24 h under microaerophilic conditions, the transformed cells were streaked onto Mueller-Hinton II agar plates containing metronidazole (1.0, 2.0, 4.0, 8.0, 16, 32, 64, and 128 mg/liter), and several single colonies were separately collected from the lowest to the highest concentrations on the metronidazole-containing plates where they were seen and spread onto metronidazole-free horse blood agar plates. The bacterial cells from each colony were harvested and diluted in brucella broth after incubation for 3 to 4 days. The culture medium was further inoculated onto metronidazole-free horse blood agar plates at least three times before being used to evaluate metronidazole susceptibility by Etest and the agar dilution method. Successful transformations and mutations were confirmed with PCR, followed by DNA sequencing analysis. For double mutation induction, amplified PCR products containing a two-base-pair insertion at position 571 (-571TA) in the *frxA* gene were introduced into transformants that contained a mutated *rdxA* PCR product. PCR fragments containing mutations in the *rdxA* gene obtained from a clinical metronidazole-resistant strain were used as positive-control PCR products. Amplified PCR products containing no mutations in the *rdxA* gene and the parental wild-type strain 26695-1 were used as a negative control. Each natural transformation was performed independently at least three times.

Nucleotide sequence accession numbers. We deposited the genome sequences of wild-type 26695-1 and metronidazole-resistant strain 26695-1MET at GenBank under accession no. CP010435 and CP010436, respectively.

RESULTS

Establishment of resistant strain. One metronidazole-resistant strain, denoted 26695-1MET, derived from the wild-type metronidazole-susceptible strain 26695-1, was obtained via exposure to low concentrations of metronidazole (up to 16.0 mg/liter) (Table 1). The final MICs were 128 mg/liter for 26695-1MET and 4 mg/liter for 26695-1.

Detection of mutations in resistant strains using next-generation sequencing. A genome-wide analysis of mutations that differentiated the two *H. pylori* strains 26695-1 and 26695-1MET was undertaken using Illumina next-generation sequencing. We mapped the short-read sequences of *H. pylori* 26695-1 and 26695-1MET to the 26695 genome, with coverage depths of 747× and 680×, respectively. A comparison of the two strains with the reference sequence 26695 identified 36 variants (19 single-nucleotide polymorphisms [SNPs] and 17 indels), of which 13 SNPs and 9 indels were shared by the two strains and were regarded as strain-specific variants that existed before the acquisition of drug resistance. However, 14 variants (6 SNPs and 8 indels) were found exclusively in the 26695-1MET strain within 7 genes, *hp0413*, *cag7* (*hp0527*), *frxA* (*hp0642*), *rdxA* (*hp0954*), *rpsU* (*hp0562*), *rrnA16S*

TABLE 1 Three mutations related to metronidazole resistance confirmed in 3 genes by PCR-based sequencing

Gene[a]	Position	Mutation type	Wild type[b]	Mutation[c]	Mutation in strain[d]: 26695-1MET	Mutation in strain[d]: 26695-1
frxA (*hp0642*)	571	Indel		TA	+	−
rdxA (*hp0954*)	3	SNP[e]	G	A	+	−
rpsU (*hp0562*)	37	SNP	G	T	+	−

[a] *frxA* encodes a NAD(P)H-flavin oxidoreductase; *rdxA* encodes an oxygen-insensitive NAD(P)H nitroreductase; *rpsU* encodes the 30S ribosomal protein S21.
[b] The wild type column shows the nucleotide or segment of nucleotide in the wild-type strain.
[c] The mutation column shows the nucleotide mutation or insertion mutations found in the mutant strain.
[d] Strain 26695-1MET is a metronidazole-resistant strain; 26695-1 is a wild-type strain (26695). +, nucleotide mutation or insertion mutations occurred; −, no nucleotide mutation or insertion mutations occurred.
[e] SNP, single-nucleotide polymorphism.

TABLE 3 PCR-based sequencing results and final MICs of successful transformants

Transformants with mutations[a]	*rdxA*[b]	*frxA*[c]	*rpsU*	Final MICs (mg/liter)
rdxA(G3A)	G3A	WT	WT	16
rdxA(G3A) + *frxA*(-571TA)	G3A	-571TA	WT	64
rdxA(C46T, G238A, G352A)	C46T, G238A, G352A	WT	WT	48
Strain 26695-1	WT	WT	WT	4.0

[a] Transformants that carried mutations in the *rdxA* gene obtained from clinical samples were used as a positive control. Wild-type strain 26695-1 was used as a negative control.
[b] G3A, the substitution of guanine to adenine at position 3 in the *rdxA* gene.
[c] WT, wild type; -571TA, two-base-pair insertion at position 571 in the *frxA* gene. C46T, G238A, and G352A, the substitution of cytosine to thymine, guanine to adenine, and guanine to adenine at position 46, 238, and 352 in *rdxA* gene, respectively.

(*hpr04*), and *rrnB16S* (*hpr07*) (see Table S2 in the supplemental material). Among them, mutations in 3 genes (*frxA*, *rdxA*, and *rpsU*) were confirmed by PCR and sequencing. The mutation G3A in *rdxA*, which is a missense mutation, changes the start codon ATG (methionine) to ATA (isoleucine). A two-base-pair frameshift insertion at position 571 (-571TA) in *frxA* created a premature stop codon (TAA). The G38T mutation in the *rpsU* gene did not create a premature stop codon; however, it was responsible for an amino acid change from an aspartic acid (D) to tyrosine (Y) at the 13th amino acid of the *rpsU* protein sequence.

Confirmation that the mutations are involved in resistance using natural transformation. To determine whether the three mutations in the three genes *frxA*, *rdxA*, and *rpsU* were necessary and sufficient to mediate metronidazole resistance, the mutated PCR products were transformed into metronidazole-susceptible *H. pylori* strain 26695-1 using natural transformation. The transformed cells were selected on Mueller-Hinton II agar plates supplemented with serial concentrations of metronidazole by using an agar dilution method (from 1.0 to 128 mg/liter by double dilution). Three candidate mutations were separately introduced into wild-type strain 26695-1 (Table 2).

The colonies from a candidate mutation in *rdxA* [named *rdxA*(G3A)]were successfully obtained from plates containing 4.0 to 8.0 mg/liter metronidazole (Table 2). At least 8 colonies from the plates with the lowest to the highest metronidazole concentrations were obtained for further evaluation of MICs, and the final MICs were 16 mg/liter for *rdxA*(G3A) (Table 3). The corresponding mutation in each transformant was confirmed with PCR-based sequencing. No mutations in the *frxA* and *rpsU* genes were confirmed in these selected transformants. We did not obtain any transformants for the candidate mutations in *frxA* and *rpsU*, even on the plates containing 2.0 mg/liter metronidazole. PCR fragments containing 3 mutations, C46T, G238A, and G352A, in the *rdxA* gene obtained from a clinical metronidazole-resistant strain (MIC, 48 mg/liter) from our previous study (39) were used as positive-control PCR products. Several colonies (named *rdxA*[C46T, G238A, and G352A]) were observed on the plates beginning with metronidazole concentrations from 4.0 to 32 mg/liter (Table 2). At least 8 colonies from these metronidazole-containing plates were obtained for further evaluation of MICs and confirmation of the mutation. The final MICs of all selected *rdxA*(C46T, G238A, and G352A) colonies were 48 mg/liter (Table 3). None of three candidate mutations in *rdxA*, *frxA*, and *rpsU* were observed in all positive-control transformants with PCR-based sequencing. To control for spontaneous mutation, the PCR products that were amplified with the same primers for the *rdxA* gene but without any mutations were introduced into the metronidazole-susceptible strain 26695-1 as a negative control. We did not observe any colonies in repeated experiments, even on the plates containing 4.0 mg/liter metronidazole (colonies were observed only on the plates containing 2.0 mg/liter metronidazole). We also used the metronidazole-susceptible parental strain 26695-1 without transformation and showed that the colonies were obtained only on the plates containing 2.0 mg/liter metronidazole.

To evaluate the synergic effects of the candidate mutations, further natural transformations were performed with the same method as that used for single transformation. Amplicons containing -571TA in *frxA* obtained from 26695-1MET were introduced into the *rdxA*(G3A) transformants, and we obtained transformants containing double mutations in the *frxA* and *rdxA* genes, which were confirmed with PCR-based sequencing. These transformants showed higher MICs (64 mg/ml) than those with single mutations.

TABLE 2 Three candidate mutations introduced into strain 26695-1 via natural transformation using the agar dilution method under metronidazole selection

PCR products for genes containing mutations[a]	Transformants recovered at maximum metronidazole concn (mg/liter) of:
frxA(-571TA)	2.0
***rdxA*(G3A)**	**8.0**
***rdxA*(C46T, G238A, G352A)**	**32**
rpsU(G37T)	2.0
rdxA (G3G)	2.0
Strain 26695-1	2.0

[a] Bold type indicates that the transformant showed resistance to metronidazole. PCR products containing three mutations C46T, G238A, and G352A in *rdxA* obtained from the clinical sample were introduced into the wild-type strain 26695-1 used as a positive control. PCR products without any mutations in *rdxA* were introduced into the wild-type strain 26695-1 and used as a negative control. Wild-type strain 26695-1 was also used as a negative control.

DISCUSSION

Metronidazole has been widely used in combination therapies with other antimicrobials to eradicate *H. pylori* and other anaerobic bacterial infections (5, 9, 10). Resistance to metronidazole, a significant cause of *H. pylori* treatment failure (11–13), is becoming increasingly common in many countries worldwide (14, 15). It is now accepted that metronidazole resistance is predominantly caused by mutations in *rdxA* (16–19) and that mutations in *frxA* enhance the resistance of *rdxA* gene mutations (20–23). However, metronidazole-resistant strains without mutations in the *rdxA* and *frxA* genes have been reported, indicating that additional genes are involved in metronidazole resistance (40–44). In this study, we successfully cultured a single metronidazole-resistant strain under the selection of low metronidazole concentrations from a susceptible strain and identified seven genes with a number of genetic variants associated with metronidazole resistance using next-generation sequencing. However, mutations in the 4 genes, including *hp0413*, *cag7*, *rrnA16S*, and *rrnB16S*, were not confirmed by PCR sequencing. This probably is because these mutations are mostly located in the repeated region of these gene sequences where next-generation sequencing (NGS) may not read well. Surprisingly, only a single sequence in addition to that in the two well-known *rdxA* and *frxA* genes related to metronidazole resistance was confirmed to contain a variant associated with resistance. Finally, we confirmed mutations in the *rdxA* and *frxA* genes known to be associated with metronidazole resistance through natural transformation experiments.

Using natural transformation experiments, we confirmed that transformants containing three mutations, C46T, G238A, and G352A, in the *rdxA* gene used as a positive control showed moderate resistance to metronidazole (MIC, 48 mg/liter). Mutation G3A in *rdxA* was successfully transformed into metronidazole-susceptible strain 26695-1, which had a low metronidazole MIC (16 mg/liter). Although the methodology has been established and used not only for experiments on metronidazole resistance (38, 45–48) but also for clarithromycin (32), tetracycline (49), fluoroquinolones (50, 51), and amoxicillin (52, 53), spontaneous mutations may have occurred under the metronidazole selection introduced during our experiment. However, no transformants were obtained with the plates containing 2.0 mg/liter metronidazole when the metronidazole-susceptible strain 26695-1 was transformed with PCR products without any mutations, which suggests that spontaneous mutations are unlikely to account for the resistant phenotype observed.

Transformants containing two mutations of -571TA in *frxA* and G3A in *rdxA* showed resistance to metronidazole with higher MICs than those with the transformants containing only G3A in *rdxA* (48 mg/liter versus 16 mg/liter, respectively). These results clearly demonstrate the presence of synergistic effects and that mutations in *frxA* contribute to the achievement of higher MICs in the presence of *rdxA* gene mutations. These findings support the hypothesis that mutations in the *rdxA* gene are the main mechanism of metronidazole resistance in *H. pylori* and that mutations in the *frxA* gene can increase the degree of resistance in the presence of mutations in the *rdxA* gene, as described in previous studies (16–23).

We were unable to obtain transformants containing a mutation at -571TA in *frxA* alone, although this mutation created a premature stop codon (TAA), which suggests that it may have caused metronidazole resistance; therefore, we hypothesize that the inactivation of *frxA* itself cannot induce metronidazole resistance. In contrast to other studies that concluded that mutations in *frxA* without mutations in *rdxA* might cause metronidazole resistance (22, 24, 40), we suggested that this may be due to other additional but uncharacterized mechanisms of metronidazole resistance that coexist with the mutations in the *frxA* gene (24).

We have identified, for the first time, the *rpsU* gene to be associated with metronidazole resistance. *rpsU* encodes a 30S ribosomal protein, S21, which is involved in protein synthesis (54–56), and was previously identified with antibiotic-resistant recombinants in *Escherichia coli* (57), which suggests that it may play a role in metronidazole resistance in *H. pylori* via an unknown mechanism. We found 2 metronidazole-resistant clinical isolates from Vietnam that had two different missense mutations in *rpsU* but not metronidazole-susceptible clinical isolates, in which one strain had mutations in *rdxA* gene and the other one did not have a mutation in either *rdxA* or *frxA* (positions G100A and G123T; T. T. Binh and Y. Yamaoka, unpublished observation). However, we were unable to obtain transformants using PCR products containing mutations in this gene. We do not know whether the transformants did not have a strong enough metronidazole-resistant phenotype to be selected under metronidazole selection when this mutation was introduced into parental strain 26695-1, because we used simple PCR products with a mutation for natural transformation, indicating the absence of selection with antibiotic cassettes (e.g., chloramphenicol selection). We are now trying to transform this candidate mutation into 26695-1 via selection by using chloramphenicol cassettes to confirm whether it can play roles in metronidazole resistance. Furthermore, our study has several limitations. We did not obtain metronidazole-resistant strains without mutations in the *frxA* and *rdxA* genes in order to confirm the presence of other mutations outside these two genes that are associated with metronidazole resistance; therefore, further work is required to identify the role of mutations in addition to those known in the *frxA* and *rdxA* genes. On the other hand, it is well known that next-generation sequencing alone cannot read the whole genome, as one contig and some sequences of the genome may not be read completely, especially in the repeated regions of the DNA sequences (58, 59). Therefore, we may have missed some other mutations in other genes that may be related to metronidazole resistance. Nonetheless, we did confirm that next-generation sequencing technology can be a useful tool for screening mutations related to drug resistance.

In conclusion, we first analyzed the genome profile for metronidazole resistance in *H. pylori* using next-generation sequencing, showing that this technology is useful for identifying mutations that differ between a susceptible and resistant strain, and it offers a significant advantage over candidate gene approaches that examine only a fraction of the genome at any one time. Our study confirms that mutations in the *rdxA* gene are mainly associated with metronidazole, whereas mutations in the *frxA* gene enhance *H. pylori* resistance exclusively in the presence of *rdxA* mutations. Finally, the ribosomal gene *rpsU* may be an additional candidate associated with metronidazole resistance.

ACKNOWLEDGMENTS

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (25293104, 24659200, and 24406015) (to Y.Y.), the Special Co-

ordination Funds for Promoting Science and Technology from the MEXT of Japan (to Y.Y.), and National Institutes of Health grants DK62813 (to Y.Y.) and GM94053 (to D.H.K.). T.T.B. is a doctoral student supported by the Japanese Government (Monbukagakusho, MEXT) Scholarship Program for 2010.

We declare no competing interests.

REFERENCES

1. **Peek RM, Jr, Blaser MJ.** 2002. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. Nat Rev Cancer **2:**28–37. http://dx.doi.org/10.1038/nrc703.
2. **Suerbaum S, Michetti P.** 2002. *Helicobacter pylori* infection. N Engl J Med **347:**1175–1186. http://dx.doi.org/10.1056/NEJMra020542.
3. **Hosking SW, Ling TK, Chung SCS, Yung MY, Cheng AF, Sung JJ, Li AKC.** 1994. Duodenal ulcer healing by eradication of *Helicobacter pylori* without anti-acid treatment: randomised controlled trial. Lancet **343:**508–510. http://dx.doi.org/10.1016/S0140-6736(94)91460-5.
4. **Takenaka R, Okada H, Kato J, Makidono C, Hori S, Kawahara Y, Miyoshi M, Yumoto E, Imagawa A, Toyokawa T, Sakaguchi K, Shiratori Y.** 2007. *Helicobacter pylori* eradication reduced the incidence of gastric cancer, especially of the intestinal type. Aliment Pharmacol Ther **25:**805–812. http://dx.doi.org/10.1111/j.1365-2036.2007.03268.x.
5. **Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ.** 2007. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. Gut **56:**772–781. http://dx.doi.org/10.1136/gut.2006.101634.
6. **Sugiyama T, Sakaki N, Kozawa H, Sato R, Fujioka T, Satoh K, Sugano K, Sekine H, Takagi A, Ajioka Y, Takizawa T, *H. pylori* Forum Gastritis Study Group.** 2002. Sensitivity of biopsy site in evaluating regression of gastric atrophy after *Helicobacter pylori* eradication treatment. Aliment Pharmacol Ther **16**(Suppl 2):S187–S190. http://dx.doi.org/10.1046/j.1365-2036.16.s2.17.x.
7. **Vannella L, Lahner E, Bordi C, Pilozzi E, Di Giulio E, Corleto VD, Osborn J, Delle Fave G, Annibale B.** 2011. Reversal of atrophic body gastritis after *H. pylori* eradication at long-term follow-up. Dig Liver Dis **43:**295–299. http://dx.doi.org/10.1016/j.dld.2010.10.012.
8. **McColl KE.** 2010. Clinical practice. *Helicobacter pylori* infection. N Engl J Med **362:**1597–1604. http://dx.doi.org/10.1056/NEJMcp1001110.
9. **Mégraud F, Lamouliatte H.** 2003. Review article: the treatment of refractory *Helicobacter pylori* infection. Aliment Pharmacol Ther **17:**1333–1343. http://dx.doi.org/10.1046/j.1365-2036.2003.01592.x.
10. **Graham DY, Fischbach L.** 2010. *Helicobacter pylori* treatment in the era of increasing antibiotic resistance. Gut **59:**1143–1153. http://dx.doi.org/10.1136/gut.2009.192757.
11. **Jenks PJ.** 2002. Causes of failure of eradication of *Helicobacter pylori*. BMJ **325:**3–4. http://dx.doi.org/10.1136/bmj.325.7354.3.
12. **Qasim A, O'Morain CA.** 2002. Review article: treatment of *Helicobacter pylori* infection and factors influencing eradication. Aliment Pharmacol Ther **16**(Suppl 1):S24–S30.
13. **Suzuki T, Matsuo K, Ito H, Sawaki A, Hirose K, Wakai K, Sato S, Nakamura T, Yamao K, Ueda R, Tajima K.** 2006. Smoking increases the treatment failure for *Helicobacter pylori* eradication. Am J Med **119:**217–224. http://dx.doi.org/10.1016/j.amjmed.2005.10.003.
14. **Fock KM, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, Lam SK, Xiao SD, Tan HJ, Wu CY, Jung HC, Hoang BH, Kachintorn U, Goh KL, Chiba T, Rani AA, Second Asia-Pacific Conference.** 2009. Second Asia-Pacific Consensus guidelines for *Helicobacter pylori* infection. J Gastroenterol Hepatol **24:**1587–1600. http://dx.doi.org/10.1111/j.1440-1746.2009.05982.x.
15. **De Francesco V, Giorgio F, Hassan C, Manes G, Vannella L, Panella C, Ierardi E, Zullo A.** 2010. Worldwide *H. pylori* antibiotic resistance: a systematic review. J Gastrointestin Liver Dis **19:**409–414.
16. **Tankovic J, Lamarque D, Delchier JC, Soussy CJ, Labigne A, Jenks PJ.** 2000. Frequent association between alteration of the *rdxA* gene and metronidazole resistance in French and North African isolates of *Helicobacter pylori*. Antimicrob Agents Chemother **44:**608–613. http://dx.doi.org/10.1128/AAC.44.3.608-613.2000.
17. **Kwon DH, Peña JA, Osato MS, Fox JG, Graham DY, Versalovic J.** 2000. Frameshift mutations in *rdxA* and metronidazole resistance in North American *Helicobacter pylori* isolates. J Antimicrob Chemother **46:**793–796. http://dx.doi.org/10.1093/jac/46.5.793.
18. **Debets-Ossenkopp YJ, Pot RG, van Westerloo DJ, Goodwin A, Vandenbroucke-Grauls CM, Berg DE, Hoffman PS, Kusters JG.** 1999. Insertion of mini-IS605 and deletion of adjacent sequences in the nitroreductase (*rdxA*) gene cause metronidazole resistance in *Helicobacter pylori* NCTC11637. Antimicrob Agents Chemother **43:**2657–2662.
19. **Goodwin A, Kersulyte D, Sisson G, Veldhuyzen van Zanten SJ, Berg DE, Hoffman PS.** 1998. Metronidazole resistance in *Helicobacter pylori* is due to null mutations in a gene (*rdxA*) that encodes an oxygen-insensitive NADPH nitroreductase. Mol Microbiol **28:**383–393. http://dx.doi.org/10.1046/j.1365-2958.1998.00806.x.
20. **De Francesco V, Zullo A, Hassan C, Giorgio F, Rosania R, Ierardi E.** 2011. Mechanisms of *Helicobacter pylori* antibiotic resistance: an updated appraisal. World J Gastrointest Pathophysiol **2:**35–41. http://dx.doi.org/10.4291/wjgp.v2.i3.35.
21. **Matteo MJ, Pérez CV, Domingo MR, Olmos M, Sanchez C, Catalano M.** 2006. DNA sequence analysis of *rdxA* and *frxA* from paired metronidazole-sensitive and -resistant *Helicobacter pylori* isolates obtained from patients with heteroresistance. Int J Antimicrob Agents **27:**152–158. http://dx.doi.org/10.1016/j.ijantimicag.2005.09.019.
22. **Kwon DH, El-Zaatari FA, Kato M, Osato MS, Reddy R, Yamaoka Y, Graham DY.** 2000. Analysis of *rdxA* and involvement of additional genes encoding NAD(P)H flavin oxidoreductase (FrxA) and ferredoxin-like protein (FdxB) in metronidazole resistance of *Helicobacter pylori*. Antimicrob Agents Chemother **44:**2133–2142. http://dx.doi.org/10.1128/AAC.44.8.2133-2142.2000.
23. **Yang YJ, Wu JJ, Sheu BS, Kao AW, Huang AH.** 2004. The *rdxA* gene plays a more major role than *frxA* gene mutation in high-level metronidazole resistance of *Helicobacter pylori* in Taiwan. Helicobacter **9:**400–407. http://dx.doi.org/10.1111/j.1083-4389.2004.00270.x.
24. **Aldana LP, Kato M, Kondo T, Nakagawa S, Zheng R, Sugiyama T, Asaka M, Kwon DH.** 2005. *In vitro* induction of resistance to metronidazole, and analysis of mutations in *rdxA* and *frxA* genes from *Helicobacter pylori* isolates. J Infect Chemother **11:**59–63. http://dx.doi.org/10.1007/s10156-004-0370-Y.
25. **Fischer W, Windhager L, Rohrer S, Zeiller M, Karnholz A, Hoffmann R, Zimmer R, Haas R.** 2010. Strain-specific genes of *Helicobacter pylori*: genome evolution driven by a novel type IV secretion system and genomic island transfer. Nucleic Acids Res **38:**6089–6101. http://dx.doi.org/10.1093/nar/gkq378.
26. **Lara-Ramírez EE, Segura-Cabrera A, Guo X, Yu G, García-Pérez CA, Rodríguez-Pérez MA.** 2011. New implications on genomic adaptation derived from the *Helicobacter pylori* genome comparison. PLoS One **6:**e17300. http://dx.doi.org/10.1371/journal.pone.0017300.
27. **Avasthi TS, Devi SH, Taylor TD, Kumar N, Baddam R, Kondo S, Suzuki Y, Lamouliatte H, Mégraud F, Ahmed N.** 2011. Genomes of two chronological isolates (*Helicobacter pylori* 2017 and 2018) of the West African *Helicobacter pylori* strain 908 obtained from a single patient. J Bacteriol **193:**3385–3386. http://dx.doi.org/10.1128/JB.05006-11.
28. **Devi SH, Taylor TD, Avasthi TS, Kondo S, Suzuki Y, Megraud F, Ahmed N.** 2010. Genome of *Helicobacter pylori* strain 908. J Bacteriol **192:**6488–6489. http://dx.doi.org/10.1128/JB.01110-10.
29. **McClain MS, Shaffer CL, Israel DA, Peek RM, Jr, Cover TL.** 2009. Genome sequence analysis of *Helicobacter pylori* strains associated with gastric ulceration and gastric cancer. BMC Genomics **10:**3. http://dx.doi.org/10.1186/1471-2164-10-3.
30. **Kawai M, Furuta Y, Yahara K, Tsuru T, Oshima K, Handa N, Takahashi N, Yoshida M, Azuma T, Hattori M, Uchiyama I, Kobayashi I.** 2011. Evolution in an oncogenic bacterial species with extreme genome plasticity: *Helicobacter pylori* East Asian genomes. BMC Microbiol **11:**104. http://dx.doi.org/10.1186/1471-2180-11-104.
31. **Suzuki R, Shiota S, Yamaoka Y.** 2012. Molecular epidemiology, population genetics, and pathogenic role of *Helicobacter pylori*. Infect Genet Evol **12:**203–213. http://dx.doi.org/10.1016/j.meegid.2011.12.002.
32. **Binh TT, Shiota S, Suzuki R, Matsuda M, Trang TTH, Kwon DH, Iwatani S, Yamaoka Y.** 2014. Discovery of novel mutations for clarithromycin resistance in *Helicobacter pylori* by using next-generation sequencing. J Antimicrob Chemother **69:**1796–1803. http://dx.doi.org/10.1093/jac/dku050.
33. **Haas CE, Nix DE, Schentag JJ.** 1990. *In vitro* selection of resistant *Helicobacter pylori*. Antimicrob Agents Chemother **34:**1637–1641. http://dx.doi.org/10.1128/AAC.34.9.1637.
34. **National Committee for Clinical Laboratory Standards.** 1999. Performance standards for antimicrobial susceptibility testing; 6th informa-

tional supplement. M100-S9. National Committee for Clinical Laboratory Standards, Wayne, PA.
35. **Utrup LJ, Flam R, Osato M, Ferraro MJ, Reller LB, Barry A, Bush K, Silliman N.** 1998. Susceptibility testing standardization and quality control ranges for *Helicobacter pylori*; abstr C-31. Prog abstr 98th Gen Meet Am Soc Microbiol, 17 to 21 May 1998, Atlanta, GA.
36. **Mégraud F, Lehours P.** 2007. *Helicobacter pylori* detection and antimicrobial susceptibility testing. Clin Microbiol Rev **20:**280–322. http://dx.doi.org/10.1128/CMR.00033-06.
37. **Ge Z, Taylor DE.** 1997. *H. pylori* DNA transformation by natural competence and electroporation. Methods Mol Med **8:**145–152.
38. **Wang Y, Roos KP, Taylor DE.** 1993. Transformation of *Helicobacter pylori* by chromosomal metronidazole resistance and by a plasmid with a selectable chloramphenicol resistance marker. J Gen Microbiol **139:**2485–2493. http://dx.doi.org/10.1099/00221287-139-10-2485.
39. **Binh TT, Shiota S, Nguyen LT, Ho DD, Hoang HH, Ta L, Trinh DT, Fujioka T, Yamaoka Y.** 2012. The incidence of primary antibiotic resistance of *Helicobacter pylori* in Vietnam. J Clin Gastroenterol **47:**233–238. http://dx.doi.org/10.1097/MCG.0b013e3182676e2b.
40. **Marais A, Bilardi C, Cantet F, Mendz GL, Mégraud F.** 2003. Characterization of the genes *rdxA* and *frxA* involved in metronidazole resistance in *Helicobacter pylori*. Res Microbiol **154:**137–144. http://dx.doi.org/10.1016/S0923-2508(03)00030-5.
41. **Kaakoush NO, Asencio C, Mégraud F, Mendz GL.** 2009. A redox basis for metronidazole resistance in *Helicobacter pylori*. Antimicrob Agents Chemother **53:**1884–1891. http://dx.doi.org/10.1128/AAC.01449-08.
42. **Moore JM, Salama NR.** 2005. Mutational analysis of metronidazole resistance in *Helicobacter pylori*. Antimicrob Agents Chemother **49:**1236–1237. http://dx.doi.org/10.1128/AAC.49.3.1236-1237.2005.
43. **Mendz GL, Mégraud F.** 2002. Is the molecular basis of metronidazole resistance in microaerophilic organisms understood? Trends Microbiol **10:**370–375. http://dx.doi.org/10.1016/S0966-842X(02)02405-8.
44. **Solcà NM, Bernasconi MV, Piffaretti JC.** 2000. Mechanism of metronidazole resistance in *Helicobacter pylori*: comparison of the *rdxA* gene sequences in 30 strains. Antimicrob Agents Chemother **44:**2207–2210. http://dx.doi.org/10.1128/AAC.44.8.2207-2210.2000.
45. **Taylor DE, Ge Z, Purych D, Lo T, Hiratsuka K.** 1997. Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. Antimicrob Agents Chemother **41:**2621–2628.
46. **Wang G, Taylor DE.** 1998. Site-specific mutations in the 23S rRNA gene of *Helicobacter pylori* confer two types of resistance to macrolide-lincosamide-streptogramin B antibiotics. Antimicrob Agents Chemother **42:**1952–1958.
47. **Ribeiro ML, Vitiello L, Miranda MC, Benvengo YH, Godoy AP, Mendonca S, Pedrazzoli J, Jr.** 2003. Mutations in the 23S rRNA gene are associated with clarithromycin resistance in *Helicobacter pylori* isolates in Brazil. Ann Clin Microbiol Antimicrob **2:**11. http://dx.doi.org/10.1186/1476-0711-2-11.
48. **Sakinc T, Baars B, Wüppenhorst N, Kist M, Huebner J, Opferkuch W.** 2012. Influence of a 23S ribosomal RNA mutation in *Helicobacter pylori* strains on the *in vitro* synergistic effect of clarithromycin and amoxicillin. BMC Res Notes **5:**603. http://dx.doi.org/10.1186/1756-0500-5-603.
49. **Gerrits MM, de Zoete MR, Arents NL, Kuipers EJ, Kusters JG.** 2002. 16S rRNA mutation-mediated tetracycline resistance in *Helicobacter pylori*. Antimicrob Agents Chemother **46:**2996–3000. http://dx.doi.org/10.1128/AAC.46.9.2996-3000.2002.
50. **Kim JM, Kim JS, Kim N, Jung HC, Song IS.** 2005. Distribution of fluoroquinolone MICs in *Helicobacter pylori* strains from Korean patients. J Antimicrob Chemother **56:**965–967. http://dx.doi.org/10.1093/jac/dki334.
51. **Moore RA, Beckthold B, Wong S, Kureishi A, Bryan LE.** 1995. Nucleotide sequence of the *gyrA* gene and characterization of ciprofloxacin-resistant mutants of *Helicobacter pylori*. Antimicrob Agents Chemother **39:**107–111. http://dx.doi.org/10.1128/AAC.39.1.107.
52. **Kwon DH, Dore MP, Kim JJ, Kato M, Lee M, Wu JY, Graham DY.** 2003. High-level β-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. Antimicrob Agents Chemother **47:**2169–2178. http://dx.doi.org/10.1128/AAC.47.7.2169-2178.2003.
53. **Gerrits MM, Schuijffel D, van Zwet AA, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG.** 2002. Alterations in penicillin-binding protein 1A confer resistance to β-lactam antibiotics in *Helicobacter pylori*. Antimicrob Agents Chemother **46:**2229–2233. http://dx.doi.org/10.1128/AAC.46.7.2229-2233.2002.
54. **Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzegerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, Venter JC.** 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature **388:**539–547. http://dx.doi.org/10.1038/41483.
55. **Held WA, Nomura M, Hershey JW.** 1974. Ribosomal protein S21 is required for full activity in the initiation of protein synthesis. Mol Gen Genet **128:**11–22. http://dx.doi.org/10.1007/BF00267291.
56. **Van Duin J, Wijnands R.** 1981. The function of ribosomal protein S21 in protein synthesis. Eur J Biochem **118:**615–619. http://dx.doi.org/10.1111/j.1432-1033.1981.tb05563.x.
57. **Bubunenko M, Baker T, Court DL.** 2007. Essentiality of ribosomal and transcription antitermination proteins analyzed by systematic gene replacement in *Escherichia coli*. J Bacteriol **189:**2844–2853. http://dx.doi.org/10.1128/JB.01713-06.
58. **Alkan C, Sajjadian S, Eichler EE.** 2011. Limitations of next-generation genome sequence assembly. Nat Methods **8:**61–65. http://dx.doi.org/10.1038/nmeth.1527.
59. **Metzker ML.** 2010. Sequencing technologies—the next generation. Nat Rev Genet **11:**31–46. http://dx.doi.org/10.1038/nrg2626.