Association of Metronidazole Resistance and Natural Competence in \textit{Helicobacter pylori}

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To study whether the capability of horizontal DNA transfer is associated with metronidazole resistance in \textit{Helicobacter pylori}, a total of 81 clinical isolates were tested for MICs of metronidazole (MTZ). The MIC assays were performed by using the E-test and reconfirmed by the agar dilution method. Natural competence assays were performed by transferring a chloramphenicol acetyltransferase cassette and a 23S rRNA gene from a clarithromycin-resistant strain (with an A-to-G mutation at nucleotide 2143) by using natural transformation. Of the 81 isolates, 65 (80.2%) were naturally competent while 16 were not. Among the 65 naturally competent strains, 39 (60%) were highly resistant to MTZ (MICs, >32 µg/ml) while only 2 of 16 (12.5%) noncompetent strains were highly MTZ resistant (P, <0.001). Therefore, there is an association between natural competence and MTZ resistance.

\textit{Helicobacter pylori} is an important pathogen of humans because it is associated with type B gastritis and peptic ulcer and is a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma of the stomach in humans (21, 22, 32). Metronidazole (MTZ) [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is a key component of widely used combination regimens for \textit{H. pylori} eradication (19). Because of the widespread use of MTZ to treat \textit{H. pylori} infections and other infectious diseases such as gynecological infections, an increase of MTZ resistance in \textit{H. pylori} strains has emerged worldwide recently. MTZ resistance is one of the major causes of treatment failure (23). MTZ resistance rates range from less than 10% to more than 80% among different geographical regions (28). Although several genes in \textit{H. pylori} have been found to be associated with MTZ resistance, the nature of the exact mechanism of the cause and spread of MTZ resistance remains unclear.

Horizontal gene transfer can occur via conjugation, transformation, or transduction, and most \textit{H. pylori} strains are naturally competent for transformation (20). The mechanism and ultimate advantage for the bacteria of natural competence is not clearly understood, although several possible biological benefits have been discussed. Generally, transformation can introduce foreign DNA, which might contain genes that play an important role in virulence and adaptation. To explore whether natural competence plays a role in the spread of a drug resistance gene(s) in \textit{H. pylori}, we studied the distribution of MTZ MICs for \textit{H. pylori} in strains with and without natural competence.

Bacterial strains. \textit{H. pylori} strains had been collected during endoscopy examinations at National Taiwan University Hospital since 1991. A total of 2,050 strains were obtained from 1991 to 2000. Multiple isolates were taken only from patients with \textit{H. pylori} treatment failure. The strains have been used for studying transmission in families (29) and MTZ resistance and clarithromycin resistance mechanisms (7, 14). Twenty strains from each year from 1996 to 1999 and a genetically well-studied strain, NTU-D1, were selected for this study (total, 81 strains). All the strains were from different patients and different families.

Laboratory tests. Natural transformation was performed by inoculating 50 µl of recipient \textit{H. pylori} cells from a –80°C stock culture onto a Columbia blood agar plate and incubating it for 2 days at 37°C (30). A heavy loop of cells (~10^9 to 10^9 cells) was then scraped from the plate and spread on a 4°C plate within a diameter of 8 to 10 mm. Incubation was continued for 5 h at 37°C. We then spotted 10 to 20 µl of DNA (0.5 to 2 µg) in Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA) or water directly onto the bacterial lawn. For determination of natural competence, a chloramphenicol acetyltransferase expression cassette (gift from D. E. Taylor, University of Alberta, Edmonton, Alberta, Canada) (31) inserted with the \textit{H. pylori} gene \textit{yjxJ} (HP0691) and a 23S rRNA gene from a clarithromycin-resistant strain (14) (with an A to G mutation at nucleotide 2143) in pCR 2.1 vector (Invitrogen, Carlsbad, Calif.) were used as DNA donors. After successful transformation, the cassette and gene were able to express chloramphenicol resistance and clarithromycin resistance phenotypes, respectively. The plates were grown for 16 to 24 h


table 1

<table>
<thead>
<tr>
<th>Competence of strains</th>
<th>MTZ MIC (µg/ml)</th>
<th>% Resistance*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>Naturally competent</td>
<td>0.004←&gt;32</td>
<td>&gt;32</td>
</tr>
<tr>
<td>(n = 65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noncompetent</td>
<td>0.025←&gt;32</td>
<td>2</td>
</tr>
<tr>
<td>(n = 16)</td>
<td></td>
<td></td>
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</tbody>
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* Breakpoints: susceptible, <4 µg/ml; intermediate, 4 to 8 µg/ml; resistant, ≥8 µg/ml.
under microaerobic conditions, and the transformants were streaked onto Columbia agar plates containing chloramphenicol (20 μg/ml) or clarithromycin (2 μg/ml), the selective antibiotics. The transformants were grown for 3 to 4 days. Natural competence in strains was determined by successful transformation of either of the two resistance markers. The strains that failed to be transformed by both DNA donors were defined as noncompetent. Noncompetent strains were further tested by electroporation with both donor DNAs by using previously described procedures (7).

MICs of MTZ were initially screened by E-testing. For E-test determinations, the plates were flooded with 100 μl of brucella broth containing 10⁵ CFU of H. pylori/ml. After drying the plates, the E-test strip (AB Biodisk, Solna, Sweden) was applied, and MICs were read 48 h later. MICs were further checked by an agar dilution method by using Columbia agar with 5% (vol/vol) sheep blood. Strain ATCC 43504 was used as a control strain in each assay. The MICs for a given strain were consistent, and variations between E-test values were less than twofold. The MICs were also stable for some strains which had been tested 3 to 4 years ago in another study (7).

These naturally competent and high-level MTZ-resistant strains were examined by pulsed-field gel electrophoresis (PFGE) as described before (26).

Of the 81 clinical isolates, those of 65 (80.2%) strains were determined as naturally competent (42 strains were success-

![Figure 1. The distribution of MICs of MTZ for 65 naturally competent (A) and 16 noncompetent (B) H. pylori strains.](image-url)
fully transformed by both DNA donors and 23 were only successfully transformed by the mutated 23S rRNA gene). Sixteen strains were determined as noncompetent due to failure of transformation by both DNA donors. However, these strains were able to successfully transform by electroporation. Of the 65 competent strains, 40 (61.5%) were resistant to MTZ, while of the 16 noncompetent strains, 4 (25%) were resistant to MTZ (Table 1). This difference is statistically significant ($P = 0.012$, Fisher’s exact test). The MICs of MTZ for all 81 H. pylori clinical isolates and the MIC distribution among naturally competent and noncompetent strains are summarized in Table 1.

The MIC at which 50% of the isolates tested were inhibited (MIC$_{50}$) and the MIC$_{90}$ for the noncompetent strains were significantly lower than those for the competent strains ($P = 0.02$; Mann-Whitney test) (Table 1 and Fig. 1). In the naturally competent group, almost all MTZ-resistant strains (39/40) had high-level MTZ resistance (MIC = $>32$ μg/ml), while in the noncompetent group, 50% (2/4) of the MTZ-resistant strains had only intermediate levels of MTZ resistance (MTZ MIC = 4 to 8 μg/ml) ($P <0.001$; Fischer’s exact test). Naturally competent strains were much more likely to have high-level MTZ resistance than noncompetent strains. By PFGE, there were two strains which were unable to be digested by NorI and NruI and two strains which were digested into small fragments. All the remaining strains revealed different PFGE patterns (Fig. 2).

H. pylori is one of the most diverse bacterial species. Independent isolates can usually be distinguished from one another by using methods such as PFGE, arbitrarily primed PCR, restriction fragment length polymorphism analysis, multilocus enzyme electrophoresis, or the sequencing of one or a few representative genes (1, 2, 10, 11, 25, 26, 27). This diversity also has been shown to exist at the genetic level in various forms, including a high rate of point mutations in conserved genes such as $ureB$ (15) and $flaA$ (24), mosaicism within genes such as $vacA$ (4), and the presence of nonconserved DNA fragments, in particular the $cag$ pathogenicity island (6, 8).

The capability of natural transformation in H. pylori would contribute to the development of genetic diversity (17) and help in the adaptation of H. pylori to changing environments and could shed light on clinically important issues of virulence and development of antibiotic resistance (5, 13). Several possible mechanisms for MTZ resistance in H. pylori merited consideration: decreased MTZ uptake or active efflux, deficiency in MTZ activation or modification, target modification or loss, and increased DNA repair or oxygen-scavenging capabilities (13). Null mutations in just a single gene, $rdaA$, are directly responsible for MTZ resistance in H. pylori (12). According to sequence analysis of the $rdaA$ genes of five pairs of MTZ-resistant and MTZ-sensitive isolates from the same person, they differed by only one or a few base substitutions. This finding indicated that MTZ resistance resulted from de novo mutation and not from gene transfer (12).

However, another study analyzed 142 strains and found the MICs showed a bimodal distribution with two distinct populations: the first included strains for which the MICs were intermediate (MIC range, 0.125 to 6 μg/ml), and the second comprised all the resistant strains (MICs, $>32$ μg/ml). The first peak suggested the presence of a still unexplained mechanism which decreases the susceptibility of H. pylori to MTZ. The second peak suggested an acquisition of a high-level genetic resistance mechanism (18) or the possibility that natural transformation processes could be involved in the mutation of $rdaA$ (because replacement of a short patch of DNA sequence [36 to 124 bp] was demonstrated) (16). Our results showed that significantly more MTZ-resistant strains appeared in the naturally competent population than in the noncompetent population. The MTZ MICs were high for naturally competent H. pylori strains, and the noncompetent strains showed intermediate MICs. These results suggest that natural competence of H. pylori may play a role in the spread of MTZ resistance.

Failure of transformation could result from the restriction barrier of H. pylori (3, 9). In our study, by using two DNA donors (including a 23S rRNA gene with an A-to-G mutation at nucleotide 2143) which only need one nucleotide exchange to turn the phenotype into clarithromycin resistance, 16 of 81 clinical isolates (19.8%) were determined as being from noncompetent strains. Even donor DNA might be digested when the restriction-modification system is employed; the fragments used should be long enough for successful recombination. Therefore, the restriction barrier was unlikely to abolish all transformants. Successful DNA transfer by using electroporation confirmed that failures of transformation were not due to restriction barriers or unsuccessful recombination. Nevertheless, the restriction barrier might also play a role in MTZ resistance due to its interference with DNA transformation, although this hypothesis needs further investigation.

In conclusion, 65 of 81 (80.2%) clinical isolates of H. pylori in our series were naturally competent while 16 strains were noncompetent, and natural competence was associated with high levels of MTZ resistance.

REFERENCES


