Mutations in penicillin-binding proteins 1, 2 and 3 are responsible for amoxicillin resistance in *Helicobacter pylori*

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Objectives: To elucidate the relationship between the mutations of penicillin-binding protein (PBP)1, PBP2 and PBP3 and amoxicillin resistance in *Helicobacter pylori*.

Methods: The mutations detected only in clinical amoxicillin-resistant strains were determined by comparison of the deduced amino acid sequences of PBP1(HP0597), PBP2(HP1556) and PBP3(HP1565) encoded by the *pbp1*, *ftsI* and *pbp2* genes, respectively, in 13 clinical *H. pylori* strains and three ATCC strains. The contribution of the mutations in PBPs was analysed by the natural transformation of the amoxicillin-susceptible strain ATCC 700392 with various combinations of the *pbp1*, *ftsI* and *pbp2* genes from the amoxicillin-resistant strain TH743 (MIC of amoxicillin: 8 mg/L).

Results: We initially identified six, four and two mutations of PBP1, PBP2 and PBP3, respectively, which were detected only in amoxicillin-resistant strains. By the natural transformation of an amoxicillin-susceptible strain TH743, we found that mutations in PBP1 and PBP3 conferred higher resistance to amoxicillin than mutations in PBP1 and PBP2, or mutations only in PBP1. Furthermore, mutations in PBP1, PBP2 and PBP3 conferred a 256-fold higher amoxicillin resistance when compared with ATCC 700392.

Conclusions: Multiple mutations in PBP2 and PBP3, in addition to mutations in PBP1, confer higher amoxicillin resistance in *H. pylori*.

Keywords: mechanisms of resistance, β-lactams, nucleotide sequencing, resistance genes, resistance genetics

Introduction

*Helicobacter pylori* is a pathogenic bacterium that causes chronic gastritis and peptic ulcers. Amoxicillin is one of the principal antimicrobial agents used in *H. pylori* infections. Amoxicillin-resistant *H. pylori* have been increasing recently and has become a major cause of eradication failure in certain areas.1

We previously analysed clinical amoxicillin-resistant *H. pylori* isolates in Japan and showed that multiple substitutions in the transpeptidase region of penicillin-binding protein 1 (PBP1) are necessary for the expression of amoxicillin resistance in *H. pylori*.2 Gerrits et al.3 also recently reported that multiple mutational changes in PBP1 are the predominant cause of amoxicillin resistance in *H. pylori*. The PBPs are peptidoglycan biosynthetic enzymes that have a transpeptidase in the C-terminal region. As β-lactams bind to the penicillin-binding motifs (SXXK, SXN and KTG) in the transpeptidase region, alterations in or around the motif possibly confer resistance due to reduced affinity to β-lactams. PBP1(HP0597), PBP2(HP1556) and PBP3(HP1565), which are encoded by the *pbp1*, *ftsI* and *pbp2* genes, respectively, are found to be high molecular PBPs in *H. pylori*;4,5 however, there are few reports showing the relationship of mutations in PBP2 and PBP3 when compared with amoxicillin resistance. In the present study, we evaluated naturally transformed mutations in the *pbp1*, *ftsI* and *pbp2* genes from a clinical amoxicillin-resistant strain to determine whether those mutations cause amoxicillin resistance.

Materials and methods

Bacterial strains

Thirteen clinical *H. pylori* strains were isolated between 1995 and 2002 at the Tokyo Medical University Hospital in Japan. *H. pylori*...
ATCC 700392, ATCC 700824 and ATCC 43504 were also used in this study.

Antimicrobial susceptibility

MICs of amoxicillin, benzylpenicillin, ceftaxime and ceftazidime were determined using the agar dilution method according to the CLSI’s instructions. Strains were considered to be resistant, low susceptible and susceptible to amoxicillin when the MIC was ≥0.5, 0.063–0.25 and ≤0.031 mg/L, respectively.

PCR and DNA sequencing

The genome DNA of *H. pylori* was isolated, as described previously. To amplify the *pbp1*, *ftsI* and *pbp2* genes, PCR was performed with Ex Taq polymerase (Takara, Kyoto, Japan) using the primers *pbp1F* (5'-TGGCAACACCTTTAATA-3') and *pbp1R* (5'-GGCACAATAAAGCTGGCA-3'), *ftsIF* (5'-TATACTGACCTAC-3') and *ftsIR* (5'-TTGGCTCAAATGGCATATTTTCA-3'), and *pbp2F* (5'-GAAACACTTGTACATCTAAACC-3') and *pbp2R* (5'-CAGAGTGAAGACCCAGGAAAT-3'), respectively. Each fragment was purified and sequenced using the method described previously.

Natural transformations

The amoxicillin-susceptible strain, ATCC 700392, was transformed using the method described by Ge and Taylor. PCR products were obtained using amplification of the *pbp1*, *ftsI* and *pbp2* genes from the amoxicillin-resistant strain, TH743, under the same conditions mentioned earlier. Transformants were selected on Brain Heart Infusion (Oxoid, Basingstoke, UK) agar plates containing 5% horse blood and 0.25, 1 or 2 mg/L benzylpenicillin, or 1 mg/L ceftriaxone. No spontaneous mutants were found on the plates used for the selection.

Results

Susceptibilities of clinical *H. pylori* isolates to β-lactams

Among the 13 isolates, TH743 and TS1289 were resistant to amoxicillin, whereas TS281 and TS1112 had low susceptibilities to amoxicillin. The remaining nine clinical isolates, ATCC 43504, ATCC 700392 and ATCC 700824 were susceptible to amoxicillin (Table 1). The amoxicillin-resistant strain TH743 also revealed high MICs of benzylpenicillin, ceftazidime and ceftriaxone (16, 64 and 32 mg/L, respectively).

Mutations of PBPs of amoxicillin-resistant *H. pylori* strains

The mutations of *pbp1*, *pbp2* and *pbp3* that are detected only in amoxicillin-resistant strains are shown in Table 1. In addition to the previously reported six mutations (A369T, V374L, S414R, L423F, N562Y and T593A) in *pbp1*, other mutations were detected in TH743; four mutations (A296V, S494H, A541M and E572G) in *pbp2* and two mutations (A499V and E536K) in *pbp3*. The mutations S414R and N562Y in *pbp1* and S494H in *pbp2* were common in the amoxicillin-resistant strains TH743 and TS1289. The mutations N562Y in *pbp1* and S494H in *pbp2* were not detected in strains TH1112 and TS281, categorized as low susceptible to amoxicillin, but mutation A369T in *pbp1* was detected in both TS1112 and TS281.

Susceptibilities of amoxicillin-resistant transformants to β-lactams

To elucidate the relationship between the mutations in PBPs and amoxicillin resistance, we determined the susceptibilities of transformants obtained by the natural transformation of amoxicillin-susceptible strain ATCC 700392 with the *pbp1*, *ftsI* and *pbp2* genes of amoxicillin-resistant strain TH743 (Table 2). The TF1a and TF1b obtained by the natural transformation with the *pbp1* gene of TH743 revealed an 8- to 16-fold higher resistance to amoxicillin, benzylpenicillin and ceftriaxone than the recipient strain. The TF2 obtained by the natural transformation with the *ftsI* gene of TH743 revealed almost equal susceptibility to amoxicillin and benzylpenicillin, but had a 4- to 8-fold higher resistance to ceftriaxone and ceftazidime when compared with the recipient strain. No transformant was obtained by the natural transformation of ATCC 700392 with the *pbp2* gene of TH743 by three repetitions of the experiments (<8.6 × 10⁻⁷ transformants/cfu/μg of DNA). TF3a and TF3b were obtained by the natural transformation of TF1a and TF1b, respectively, with the *ftsI* gene of TH743; TF4a and TF4b were obtained by the natural transformation of TF1a and TF1b, respectively, with the *pbp2* gene of TH743. Compared with the susceptibilities of TF1a and TF1b, TF3a and TF3b were revealed to be almost equally resistant to amoxicillin and benzylpenicillin, and TF4a and TF4b were 4- to 8- and 2-fold more resistant to amoxicillin and benzylpenicillin, respectively. These transformants revealed almost equal susceptibility to ceftazidime and ceftriaxone when compared with the recipient strain. TF5 obtained by the transformation of TF3a with the *pbp1*, *ftsI* and *pbp2* genes of TH743 demonstrated 16-, 8-, 2- and 2-fold higher resistance to amoxicillin, benzylpenicillin, ceftazidime and ceftriaxone, respectively, than the recipient strain TF3a. That is, TF5 was 256-fold more resistant to amoxicillin than the original recipient strain, ATCC 700392.

Mutations of PBPs in amoxicillin-resistant transformants

The mutations of *pbp1*, *pbp2* and *pbp3* detected in the transformants are shown in Table 2. Among the six mutations in *pbp1* of TH743, four mutations were introduced in TF1a and TF1b, whereas the remaining two mutations (A369T and V374L) were not detected in TF1b. All four mutations in *pbp2* and a mutation of *pbp3* were introduced in all transformants. No unexpected mutation in the *pbp1*, *ftsI* and *pbp2* genes was detected in transformants obtained in this study.

Discussion

The correlation between the mutations in *pbp2* and *pbp3* and amoxicillin resistance has not been elucidated, although it has been reported that the mutations in *pbp1* confer amoxicillin resistance in *H. pylori*. Here, we show for the first time the mutations in *pbp2* and *pbp3* that confer heightened amoxicillin resistance in *H. pylori*. In addition to the mutations in *pbp1* previously reported, we identified the mutations in *pbp2* and *pbp3* that were
Mutations of PBPs in amoxicillin-resistant *H. pylori*

Table 1. Mutations in PBP1, PBP2 and PBP3 occurring in clinical *H. pylori* isolates with resistance and low susceptibilities to amoxicillin

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amoxicillin MIC (mg/L)</th>
<th>PBP1, encoded by <em>pbp1</em></th>
<th>PBP2, encoded by <em>ftsI</em></th>
<th>PBP3, encoded by <em>pbp2</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>369 374 414 423 562 593</td>
<td>296 494 541 572 499 536</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin-susceptible strains (n = 12)</td>
<td>≤0.031</td>
<td>Ala Val Ser Leu Asn Thr Ala Asn/Ser Ala/Val Glu Ala Glu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TH743</td>
<td>8</td>
<td>Thr Leu Arg Phe Tyr Ala Val His Met Gly Val</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS1289</td>
<td>0.5</td>
<td>. . Arg . Tyr . . . . . . . . . . Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS1112</td>
<td>0.125</td>
<td>Thr . . . . . . . . . . . . . . . . . . . .</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS281</td>
<td>0.125</td>
<td>Thr . Arg . . . . . . . . . . . . . . . . . . . .</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

detected only in amoxicillin-resistant or low-susceptibility strains. To demonstrate the contribution of the mutations in PBP1, PBP2 and PBP3 to amoxicillin resistance, the amoxicillin-susceptible strain ATCC 700392 was transformed with various combinations of the *pbp1*, *ftsI* and *pbp2* genes from the amoxicillin-resistant strain TH743. Although transformants with mutations of PBP1 revealed low susceptibilities to amoxicillin, TF2-containing PBP2 mutations were susceptible to amoxicillin. These data suggest that the mutation of PBP2 was not critical for the first step in a change in the susceptibility to amoxicillin in *H. pylori*. However, the level of resistance to ceftriaxone and ceftazidime was increased by the mutations of PBP2. DeLoney and Schiller\(^5\) reported that the primary function of PBP with molecular masses of 63 kDa (PBP63) in *H. pylori* is in septum formation and that ceftriaxone preferentially binds to PBP63. Using an estimate of the molecular mass and function, PBP63 corresponded to PBP2 encoded by the *ftsI* gene.\(^4\) These reports support our data, showing that the mutations in PBP2 confer resistance to ceftriaxone and ceftazidime in *H. pylori*.

DeLoney and Schiller\(^5\) also report amoxicillin bound to PBP60 (corresponding to PBP3 encoded by the *pbp2* gene) very strongly compared with other PBPs. Although no transformants possessing PBP3 mutations were obtained in this study, we did

Table 2. Susceptibility to β-lactams and mutations in PBPs of amoxicillin-resistant transformants

<table>
<thead>
<tr>
<th>Recipient/donor DNA, strain</th>
<th>MIC (mg/L)</th>
<th>Amino acid position of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amoxicillin benzylpenicillin ceftazidime ceftriaxone</td>
<td>PBP1 PBP2 PBP3</td>
</tr>
<tr>
<td></td>
<td>369 374 414 423 562 593 296 494 541 572 499 536</td>
<td></td>
</tr>
<tr>
<td>ATCC 700392</td>
<td>0.016 0.016 1 0.25</td>
<td>Ala Val Ser Leu Asn Thr Ala Ser Ala Glu Ala</td>
</tr>
<tr>
<td>TH743</td>
<td>8 16 64 32</td>
<td>Thr Leu Arg Phe Tyr Ala Val His Met Gly Val</td>
</tr>
<tr>
<td>ATCC 700392/TH743 <em>pbp1</em></td>
<td>0.125 0.25 2 2</td>
<td>. . Arg Phe Tyr Ala . . . . . .</td>
</tr>
<tr>
<td>TF1a</td>
<td>0.25 0.25 4 2</td>
<td>Thr Leu Arg Phe Tyr Ala . . . . . .</td>
</tr>
<tr>
<td>ATCC 700392/TH743 <em>ftsI</em></td>
<td>0.031 0.031 4 2</td>
<td>. . . . . . . . . . Val His Met Gly .</td>
</tr>
<tr>
<td>TF1b</td>
<td>0.25 0.25 4 2</td>
<td>. . . . . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>TF2</td>
<td>0.25 0.5 4 4</td>
<td>. . Arg Phe Tyr Ala Val His Met Gly .</td>
</tr>
<tr>
<td>TF3a</td>
<td>0.125 0.25 4 4</td>
<td>Thr Leu Arg Phe Tyr Ala Val His Met Gly .</td>
</tr>
<tr>
<td>TF3b(^a)</td>
<td>0.25 0.5 4 4</td>
<td>. . . . . . . . . . . . . . . . . . . .</td>
</tr>
<tr>
<td>TF4a</td>
<td>1 0.5 2 2</td>
<td>. . Arg Phe Tyr Ala . . . . . . Val</td>
</tr>
<tr>
<td>TF4b(^a)</td>
<td>1 0.5 4 2</td>
<td>Thr Leu Arg Phe Tyr Ala . . . . . . Val</td>
</tr>
<tr>
<td>TF5</td>
<td>4 4 8 8</td>
<td>. . Arg Phe Tyr Ala Val His Met Gly Val</td>
</tr>
</tbody>
</table>

\(^a\)TF3b and TF4b were obtained by transformation of TF1b with the *ftsI* and *pbp2* genes from strain TH743, respectively.
obtain TF4a- and TF4b-possessing mutations in PBP1 and PBP3, which revealed higher resistance to amoxicillin than TF1a and TF1b. In addition, the levels of resistance to amoxicillin in TF4a and TF4b were higher than in TF3a and TF3b. These data indicate that mutations in PBP3 are more significant in increasing the level of amoxicillin resistance than those in PBP2. Furthermore, transformants possessing mutations in PBP1, PBP2 and PBP3 revealed a 256-fold higher resistance to amoxicillin than the recipient strain ATCC 700392. This suggests that multiple mutations in PBP1, PBP2 and PBP3 contribute to a greater increase in the level of amoxicillin resistance. As four mutations in PBP2 and one mutation in PBP3 were introduced in all transformants analysed in this study, these mutations possibly confer β-lactam resistance. Further investigation is necessary to determine the relationship between individual mutations and amoxicillin resistance.

The level of resistance to amoxicillin in those transformants that had mutations in PBP1, PBP2 and PBP3 was slightly lower than in the original clinical amoxicillin-resistant TH743 strain. It is possible that other factors, such as mutations in the porin proteins, function as an additional mechanism for high-level amoxicillin resistance in clinical H. pylori isolates.

The results of our study show that multiple mutations in PBP2 and PBP3, in addition to those in PBP1, confer amoxicillin resistance in H. pylori.

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Transparency declarations

None to declare.

References