A change in PBP1 is involved in amoxicillin resistance of clinical isolates of *Helicobacter pylori*

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Reports on the isolation of amoxicillin-resistant *Helicobacter pylori* are increasing worldwide, which may cause serious problems in eradication therapy. To elucidate the mechanism of amoxicillin resistance of *H. pylori*, penicillin-binding proteins (PBPs) of amoxicillin-resistant strains isolated in Korea were analysed. Three PBPs (66, 63 and 60 kDa) were identified in both amoxicillin-resistant and -susceptible strains using biotinylated ampicillin, and the PBP profiles were very similar irrespective of the difference in amoxicillin susceptibility. We obtained clones with moderate resistance from an amoxicillin-susceptible strain, HPK5, by transformation with genomic DNA from an amoxicillin-resistant strain, HPA116. In a resistance-induced clone, HPO1, the affinity of PBP1 for amoxicillin was reduced. The *pbp1* genes from HPA116, HPO1 and HPK5 were cloned and sequenced. The nucleotide sequences of *pbp1* from HPA116 and HPO1 were almost identical, whereas that of HPK5 was quite different. Both the ORFs of HPA116 and HPO1 *pbp1* have four substitutions and one insertion of amino acid residues compared with those of HPK5 and other sensitive strains. All the mutations, except one, are in the C-terminal half of the 659-amino-acid sequence containing the penicillin-binding modules. DNA fragments containing either full-length or a C-terminal half of *pbp1* could transform HPK5 to have resistance, indicating that changes in the penicillin-binding core of PBP1 are involved in the amoxicillin resistance of *H. pylori* isolated in Korea.

Introduction

*Helicobacter pylori* is a Gram-negative spiral bacillus that was isolated from the stomach of a patient with chronic active gastritis by Marshall & Warren.1 *H. pylori* is considered to be an important factor in peptic ulcers since eradication therapy significantly lowers the recurrence rate for *H. pylori*-positive peptic ulcers.2 In addition, there is aetiological evidence that the risk of gastric cancer is increased by infection with *H. pylori*, suggesting its association with gastric cancer.3 Moreover, cure of *H. pylori* infection has been associated with regression of gastric mucosa-associated lymphoid tissue lymphoma.4,5

Currently available eradication regimens for *H. pylori* are triple drug combination regimens comprising a proton pump inhibitor and two antibiotic drugs, and eradication rates of 70–90% are obtained using these regimens.6 Although clarithromycin and metronidazole are commonly used antibiotics for eradication treatment, *H. pylori* bacteria easily become resistant to these drugs, and the occurrence of such resistant strains decreases the eradication rate.7 Regarding the mechanism of acquiring resistance to clarithromycin, genetic analysis has shown that the resistance develops through a point mutation of the 23S ribosomal RNA gene.8 Metronidazole resistance of *H. pylori* has been shown to be due to the mutational inactivation of the *rdxA* gene, which encodes...
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Nitroreductase, an enzyme that reduces metronidazole to an active form.\textsuperscript{10,11}

*H. pylori* has been considered to seldom become resistant to amoxicillin. In recent years, however, reports on the isolation of resistant strains are increasing worldwide, with a high frequency in particular geographical regions.\textsuperscript{12-16} Reports are available concluding that amoxicillin resistance is a cause of unsuccessful eradication.\textsuperscript{17,18} If amoxicillin-resistant strains of *H. pylori* were to spread further, serious problems would arise, resulting in increasing eradication failures.

Gram-negative bacteria often become resistant to β-lactams by acquiring β-lactamase genes on chromosomes or plasmids.\textsuperscript{19} It appears, however, that β-lactamase is not produced by any of the amoxicillin-resistant clinical isolates of *H. pylori* that have been reported to date.\textsuperscript{12-15} Resistance to β-lactams in Gram-negative bacteria may also be associated with mutations of penicillin-binding proteins (PBPs), changes in drug permeability, multiple-drug efflux mechanisms, etc. Reports on resistant strains from clinical isolates as well as resistance-induced strains in vitro have suggested that changes in PBPs are involved.\textsuperscript{20-22} In the present study, we show that changes in PBP1 are involved in the amoxicillin resistance of *H. pylori* isolated in Korea.

Materials and methods

Bacterial strains and cultures

*H. pylori* strains used in this study and their origins are listed in Table 1. Strains of HPA series were isolated in Kosin Medical Center, Pusan, Korea, whereas HPK5 and CPY3401 were isolated in Yamaguchi University Hospital, Ube, Japan. In Kosin Medical Center, amoxicillin-resistant *H. pylori* were isolated from gastric cancer patients with a prevalence of 11.2%.\textsuperscript{28} Until processed, the strains were stored at –80°C in Brucella broth (Difco Laboratories, Detroit, MI, USA) supplemented with 20% glycerol.

The strains were cultured in Brucella broth or Brucella agar supplemented with 3% horse serum (Gibco Bethesda Research Laboratories, Grand Island, NY, USA) and 0.1% β-cyclodextrin (Wako Pure Chemical Industries, Osaka, Japan) at 37°C under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) with humidity.

MIC determination

In conformity to the NCCLS standard,\textsuperscript{29} MIC was determined by the agar dilution method using Mueller–Hinton medium supplemented with 5% sheep blood (Nihon BioTest Co., Tokyo, Japan). Bacterial suspensions equivalent to a 2.0 MacFarland standard (containing 1 × 10⁷–1 × 10⁸ cfu/mL) were prepared in saline from a 72 h subculture from a blood agar plate. The inoculum (1–3 µL per spot) was plated on serial doubling dilutions of amoxicillin on the plate.

Detection of β-lactamase

The production of β-lactamase by *H. pylori* strains was tested by the chromogenic cephalosporin method using the P/C Ase test kit (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) as directed by the manufacturer.

Preparation of bacterial membranes

Cells grown in 300 mL of Brucella broth were harvested at early log phase (OD₅₉₀ = 0.4), and membrane fractions were obtained by the method of DeLoney & Schiller.\textsuperscript{30} In brief, the cells were washed twice with 50 mM sodium phosphate buffer, pH 7.2, and suspended in 10 mM phosphate-buffered saline (PBS), pH 7.2. Lysozyme (1 mg/5 mL bacterial suspension) was reacted at room temperature for 20 min and the mixture was then sonicated until most of the cells were

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin/reference</th>
<th>Disease</th>
<th>MIC (mg/L)</th>
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<tbody>
<tr>
<td>HPA116</td>
<td>Korea</td>
<td>gastric cancer</td>
<td>8</td>
</tr>
<tr>
<td>HPA175</td>
<td>Korea</td>
<td>gastric ulcer</td>
<td>8</td>
</tr>
<tr>
<td>HPA173</td>
<td>Korea</td>
<td>gastric cancer</td>
<td>0.063</td>
</tr>
<tr>
<td>HPA224</td>
<td>Korea</td>
<td>gastric cancer</td>
<td>0.031</td>
</tr>
<tr>
<td>HPA397</td>
<td>Korea</td>
<td>gastric cancer</td>
<td>0.063</td>
</tr>
<tr>
<td>HPA527</td>
<td>Korea</td>
<td>gastric ulcer</td>
<td>0.016</td>
</tr>
<tr>
<td>CPY3401</td>
<td>Japan\textsuperscript{21}</td>
<td>duodenal ulcer</td>
<td>0.031</td>
</tr>
<tr>
<td>HPK5</td>
<td>Japan\textsuperscript{24}</td>
<td>gastric ulcer</td>
<td>0.031</td>
</tr>
<tr>
<td>26695</td>
<td>United Kingdom\textsuperscript{25}</td>
<td>gastritis</td>
<td>0.016</td>
</tr>
<tr>
<td>ATCC 43504</td>
<td>Australia\textsuperscript{26}</td>
<td>gastritis</td>
<td>0.016</td>
</tr>
<tr>
<td>SS1</td>
<td>Australia\textsuperscript{27}</td>
<td>duodenal ulcer</td>
<td>0.016</td>
</tr>
<tr>
<td>HPO1</td>
<td>HPK5 transformant/this study</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>
disrupted, as visualized microscopically. Non-disrupted cells were removed by centrifugation at 8000g for 15 min, and the supernatant was subjected to centrifugation at 100 000g for 45 min. The resultant pellet consisted of both inner and outer membranes. The membranes were washed with 0.1 M sodium phosphate buffer, pH 7.2, resuspended in the same buffer and adjusted to a final protein concentration of 10–20 mg/mL using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were preserved at −80°C until use.

**BIO-AMP labelling of PBPs**

Biotin-labelled ampicillin (BIO-AMP) was prepared by the method of Dargis & Malouin.31 Briefly, ampicillin (Meiji Seika Ltd, Tokyo, Japan) and biotinamidocaproic acid 3-sulpho-N-hydroxysuccinimide ester (Pierce Chemical, Rockford, IL, USA) in a 1:5 molar ratio were dissolved in 0.1 M sodium phosphate buffer, pH 7.2, and incubated at room temperature with gentle stirring for 30 min. The reaction was stopped by the addition of a 10-fold molar excess of glycine, and incubated for a further 30 min with gentle stirring. BIO-AMP was preserved in aliquots at −80°C until use.

PBPs of *H. pylori* were labelled with BIO-AMP by incubating 200 µg of total membrane proteins and 10 mg/L of labelled ampicillin in a final volume of 100 µL (1 µg of labelled ampicillin per 200 µg of membrane protein) at room temperature for 20 min. The labelled membranes were washed twice and centrifuged, and the inner membrane proteins in the pellet were solubilized in 1% N-lauroylsarcosine (Sigma, St Louis, MO, USA) for 20 min. The insoluble membranes were then removed by centrifugation, dissolved in the SDS–PAGE sample buffer and boiled for 2 min. The samples were subjected to 10% SDS–PAGE, and the separated proteins were transferred on to a polyvinylidene fluoride (PVDF) membrane using a semi-dry blotting apparatus (Bio-Rad Laboratories). After being blocked with skimmed milk, the biotinylated PBPs were bound to streptavidin–peroxidase complex and visualized with chemiluminescence using ECL (Amersham Biosciences Corp., Piscataway, NJ, USA).

**Amoxicillin competition assay**

To determine the affinity of PBP for amoxicillin, membrane fractions were pre-incubated with amoxicillin at concentrations of 0.05, 0.5, 5 and 50 mg/mL at room temperature for 10 min, and then BIO-AMP was added (10 mg/L of labelled ampicillin, 1 µg of labelled ampicillin per 200 µg of membrane protein) followed by incubation at room temperature for 20 min. Subsequently, the biotin-labelled PBPs were visualized with chemiluminescence as described above, and the affinity was rated on the basis of label intensity.

**Transformation of *H. pylori* with genomic or PCR-amplified DNA**

Genomic DNA was isolated using ISOPLANT (Nippon Gene Co., Ltd, Tokyo, Japan) according to the method described by the manufacturer. The 2199 bp fragment ranging from 121 nt upstream to 98 nt downstream of *pbp1* (full-length *pbp1*) and the 1067 bp fragment ranging from 1012 nt downstream of the initiation codon to 98 nt downstream of *pbp1* (C-terminal half of *pbp1*) were amplified by PCR using HPA116 or ATCC 43504 as a template.

Five micrograms of the genomic DNA or 1 µg of the PCR product purified by agarose gel electrophoresis was added to 500 µL of a culture of strain HPK5 in the early log phase (OD₉₀₀ = 0.4) followed by microaerobic culture for 12 h. After harvesting, the cells were spread over a Brucella agar plate supplemented with amoxicillin (1 mg/L) and incubated microaerobically for 72 h.

**DNA sequence determination and analysis**

The full-length *pbp1* gene was amplified by PCR from HPA116, HPK5 and HPO1 using KOD plus DNA polymerase (Toyobo Co., Ltd, Osaka, Japan). Enzymic clean-up of the PCR product was performed using exonuclease I and shrimp alkaline phosphatase (Amersham Biosciences Corp.) to remove excess oligonucleotide primers and dNTPs. The purified PCR products were sequenced directly using the BigDye terminator sequencing kits and ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Mutations were confirmed by reamplification and sequencing at least three times. The resulting DNA sequences and deduced amino acid sequences were aligned with a Clustal W program.32

**Nucleotide sequence accession numbers**

The nucleotide sequences for the *pbp1* gene of HPA116, HPO1 and HPK5 determined in this study are listed in DDBJ/EMBL/GenBank database under accession nos AB075014, AB075015 and AB075016, respectively.

**Results**

**MIC of amoxicillin and determination of β-lactamase**

MICs of amoxicillin for *H. pylori* strains from various sources are shown in Table 1. The MIC for two strains, HPA116 and HPA175, was more than 100-fold higher than that for the other strains. The activity of β-lactamase was detected in neither the amoxicillin-resistant strains, HPA116 and HPA175, nor the amoxicillin-susceptible strains.
Detection of PBPs

Since no β-lactamase activity was detected in the amoxicillin-resistant strains, we analysed PBPs of *H. pylori*. Membranes from amoxicillin-resistant strains, HPA116 and HPA175, and an amoxicillin-susceptible strain, ATCC 43504, were labelled with BIO-AMP and subjected to SDS–PAGE (Figure 1). Three distinct bands were detected together with several faint bands in all the preparations. The three major bands disappeared when labelling was carried out in the presence of competing ampicillin, and were identified as PBP1 (66 kDa), PBP2 (63 kDa) and PBP3 (60 kDa). Other bands, including a 31 kDa band in HPA116, were not affected by the presence of ampicillin. It should be noted that the 31 kDa band was not observed with a different membrane preparation from the same strain (Figure 2a).

PBP profiles of amoxicillin-resistant and -susceptible strains

Two amoxicillin-resistant and nine amoxicillin-susceptible strains were compared in terms of their PBP profiles (Figure 2). No major difference was observed between the resistant and susceptible strains from Korea and other countries, although there were some variations of PBPs in the apparent molecular weight.

Figure 1. PBP profiles of *H. pylori* detected by BIO-AMP. Membrane proteins from a log phase culture of *H. pylori* strains were incubated with BIO-AMP, separated by SDS–PAGE (10%) and visualized on western blots by chemiluminescence. In the right-hand three lanes of the figure, *H. pylori* membrane fractions were pre-incubated with non-labelled ampicillin (50 µg/L) prior to labelling with BIO-AMP. MICs for the strains tested were as follows: HPA116, 8 mg/L; HPA175, 8 mg/L; ATCC 43504, 0.016 mg/L. The molecular mass markers (M) are on the left. Experiments were performed in duplicate and the same results were observed.

Figure 2. PBP profiles of amoxicillin-resistant and -susceptible strains. PBP profiles were compared between amoxicillin-sensitive and -resistant strains isolated in Korea (a) and in other countries (b). *H. pylori* membrane fractions were incubated with BIO-AMP, separated by SDS–PAGE and visualized on western blots by chemiluminescence. Strains HPA116 and HPA175 were amoxicillin resistant and their MICs were 8 mg/L. The other strains were amoxicillin susceptible and MICs for these strains were as follows: HPA527, 0.016 mg/L; HPA224, 0.031 mg/L; HPA173, 0.063 mg/L; HPA397, 0.063 mg/L; HPK5, 0.031 mg/L; CPY3401, 0.031 mg/L; ATCC 43504, 0.016 mg/L; 26695, 0.016 mg/L; SS1, 0.016 mg/L. Molecular mass markers (M) are on the left. Experiments were performed in duplicate and the same results were observed.

Construction of amoxicillin-resistant clones by transformation

Twenty-six amoxicillin-resistant clones were obtained by transforming a susceptible strain, HPK5, with genomic DNA (5 µg) of HPA116, whereas no resistant clones appeared when genomic DNA of a susceptible strain, ATCC 43504, was used. The MIC for resistant clones was 2 mg/L of amoxicillin, a level 64-fold greater than that for HPK5 (0.031 mg/L), but quarter the MIC for HPA116 (8 mg/L).

Membrane fractions were prepared from HPK5, HPA116 and five transformants, and their PBP profiles were compared (Figure 3). The HPK5-derived amoxicillin-resistant transformants showed very similar PBP profiles to HPK5 and HPA116.
PBPs in clinically isolated amoxicillin-resistant *H. pylori*

**Affinity of PBPs for amoxicillin**

The affinity of PBPs for amoxicillin was determined on the basis of competition between BIO-AMP and amoxicillin (Figure 4). In the susceptible strain HPK5, labelling of PBPs was significantly reduced by increasing the concentration of amoxicillin. In contrast, in an amoxicillin-resistant transformant, HPO1, labelling of PBP1, but not PBP2 and PBP3, was not inhibited by amoxicillin. These results indicate that the affinity of PBP1 in HPO1 is considerably decreased compared with that of amoxicillin-susceptible HPK5. Similar results were obtained with the clinically isolated amoxicillin-resistant strain HPA116 (data not shown).

**DNA sequencing of the pbp1 gene**

To determine whether the reduced affinity of PBP1 for amoxicillin is due to mutation in the *pbp1* gene, the full-length *pbp1* gene from HPA116, HPK5 and HPO1 was cloned by PCR and sequenced. The nucleotide sequence of *pbp1* from HPO1 was essentially the same as that of HPA116, but quite different from that of HPK5. Only amino acid position 121 was changed from Leu in HPA116 to Ile in HPO1. The deduced amino acid sequences of PBP1 in these strains were aligned with those of 69A,22 69A/AMX,22 2669525 and J99.33 The amino acid changes conserved in all the amoxicillin-resistant strains that differ in both HPA116 and HPO1 are summarized in Table 2. The amoxicillin-resistant strains HPA116 and HPO1 have four amino acid substitutions and one insertion at positions conserved in the amoxicillin-susceptible strains including HPK5. All the mutations of HPA116/HPO1, except one at Ala-69, were found in the C-terminal half of PBP1, which has penicillin-binding modules for acyl serine transpeptidase.34 It should be noted that none of the mutations in HPA116/HPO1 is shared by the *in vitro*-induced resistant strain 69A/AMX.22

**Transformation using PCR fragments of the pbp1 gene**

To confirm whether these mutations were indeed responsible for amoxicillin resistance, we amplified the full-length *pbp1* DNA, as well as a C-terminal half of the *pbp1* gene containing the penicillin-binding modules, by PCR from the amoxicillin-resistant strain HPA116 (MIC of 8 mg/L), and transformed them into the amoxicillin-susceptible strain HPK5 (MIC of 0.03 mg/L). For the control, DNA fragments from an amoxicillin-susceptible strain, ATCC 43504, were used. DNA fragments containing either full-length or a C-terminal half of *pbp1* could transform HPK5 to have resistance, whereas no resistant clones appeared in the control, indicating that changes in the penicillin-binding module of PBP1 are involved in the amoxicillin resistance.

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Discussion

The PBPs are a set of enzymes involved in the biosynthesis of the peptidoglycan layer of the bacterial cell wall. Three PBPs in \textit{H. pylori}, PBP1 (66 kDa), PBP2 (63 kDa) and PBP3 (60 kDa) described here, were first reported by Ikeda et al.,\textsuperscript{35} followed by several authors including DeLoney et al.\textsuperscript{21,30} and Krishnamurthy et al.\textsuperscript{36} The latter authors reported that PBP1, PBP2 and PBP3 correspond to HP0597 (PBP1 homologue of \textit{Escherichia coli}), HP1556 (FtsI homologue) and HP1565 (PBP2 homologue) in the whole genome sequence of \textit{H. pylori} 26695.\textsuperscript{36} These high molecular weight PBPs are bifunctional with transglycosylase and DD-transpeptidase activities in a single polypeptide, and the latter has penicillin-binding modules as analysed by Goffin & Ghuysen.\textsuperscript{34} Minor activities in a single polypeptide, and the latter has penicillin-binding modules amplified from the amoxicillin-resistant strain (MIC 8 mg/L) to transform an amoxicillin-susceptible \textit{H. pylori} strain (MIC 0.03 mg/L) to produce an amoxicillin-resistant transformant (MIC 2 mg/L). As pointed out by DeLoney & Schiller,\textsuperscript{21} the resistance was acquired not only by the change in drug permeational barrier to \β-lactam antibiotics in the resistant clone. Similarly, Paul et al.\textsuperscript{22} obtained a stable amoxicillin-resistant clone (MIC 4–8 mg/L) from a susceptible strain (MIC 0.03–0.06 mg/L) after 39 passages by gradually increasing the concentration of amoxicillin. The PBP1 labelling of the amoxicillin-resistant clone by biotinylated amoxicillin was significantly decreased because of its reduced affinity for amoxicillin. In addition, they showed that amoxicillin resistance is due in part to an increased diffusional barrier to \β-lactam antibiotics in the resistant clone. In our experiment, the MIC of amoxicillin for the amoxicillin-resistant clinical isolate was 8 mg/L, whereas that for the resistance-acquired transformant was 2 mg/L. As in the case of amoxicillin-resistant strains produced \textit{in vitro},\textsuperscript{22} only a part of the resistant phenotype could be transformed. As pointed out by DeLoney & Schiller,\textsuperscript{21} the resistance was acquired not only by the change in PBP1, which reduced the affinity for amoxicillin, but also by the change in drug permeability.

Table 2. Amino acid changes in the \textit{pbp1} gene in amoxicillin-resistant and -susceptible strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Amoxicillin susceptibility</th>
<th>Amino acid at position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>69</td>
</tr>
<tr>
<td>HPA116</td>
<td>resistant</td>
<td>V</td>
</tr>
<tr>
<td>HPO1</td>
<td>resistant</td>
<td>V</td>
</tr>
<tr>
<td>HPK5</td>
<td>susceptible</td>
<td>A</td>
</tr>
<tr>
<td>26695</td>
<td>susceptible</td>
<td>A</td>
</tr>
<tr>
<td>999</td>
<td>susceptible</td>
<td>A</td>
</tr>
<tr>
<td>69A</td>
<td>susceptible</td>
<td>A</td>
</tr>
<tr>
<td>69A/AMX\textsuperscript{c}</td>
<td>resistant</td>
<td>A</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Position 556 is the centre of the conserved KTG sequence in motif 3.
H. pylori involved in acquiring moderate amoxicillin resistance by affinity for amoxicillin. Motifs necessary to develop genetic diagnosis for amoxicillin-resistant strains by genetic transformation. We believe that future studies will proceed to analyse other clones as well and hopefully determine other mechanisms such as mutations leading to reduced drug uptake.

We identified five mutations in pbp1 of amoxicillin-resistant strain HPA116 (Table 2). All these mutations are also found in pbp1 of HPO1 at positions conserved not only in pbp1 of amoxicillin-susceptible strains HPK5, 69A, 26695 and J99, but also in pbp1 of an amoxicillin-resistant strain, 69A/AMX2, isolated in vitrò.22 The transpeptidase domain of PBPI of H. pylori 26695 has a catalytic centre consisting of motif 1 (SXXK at 368–371), motif 2 (SLN at 433–435) and motif 3 (KGG at 555–557) when aligned with the sequence of E. coli PBPI.34 Interestingly, the amoxicillin-resistant HPA116/HPO1 has a KGG sequence in motif 3 instead of the KTG sequence conserved in other strains. Although the KGG sequence is found in some PBPs of other classes, the KTG sequence in motif 3 is invariably found in PBPI so far sequenced.35

In conclusion, we demonstrated that changes in PBPI are involved in acquiring moderate amoxicillin resistance by H. pylori, and that this phenotype is transferable to other strains by genetic transformation. We believe that future studies are necessary to develop genetic diagnosis for amoxicillin resistance of H. pylori based on mutations in pbp1 as well as to establish a new eradication therapy for amoxicillin-resistant bacteria.

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