

## Alterations in Penicillin-Binding Protein 1A Confer Resistance to $\beta$ -Lactam Antibiotics in *Helicobacter pylori*

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**Most *Helicobacter pylori* strains are susceptible to amoxicillin, an important component of combination therapies for *H. pylori* eradication. The isolation and initial characterization of the first reported stable amoxicillin-resistant clinical *H. pylori* isolate (the Hardenberg strain) have been published previously, but the underlying resistance mechanism was not described. Here we present evidence that the  $\beta$ -lactam resistance of the Hardenberg strain results from a single amino acid substitution in HP0597, a penicillin-binding protein 1A (PBP1A) homolog of *Escherichia coli*. Replacement of the wild-type HP0597 (*pbp1A*) gene of the amoxicillin-sensitive ( $\text{Amx}^s$ ) *H. pylori* strain 1061 by the Hardenberg *pbp1A* gene resulted in a 100-fold increase in the MIC of amoxicillin. Sequence analysis of *pbp1A* of the Hardenberg strain, the  $\text{Amx}^s$  *H. pylori* strain 1061, and four amoxicillin-resistant ( $\text{Amx}^r$ ) 1061 transformants revealed a few amino acid substitutions, of which only a single Ser<sub>414</sub>→Arg substitution was involved in amoxicillin resistance. Although we cannot exclude that mutations in other genes are required for high-level amoxicillin resistance of the Hardenberg strain, this amino acid substitution in PBP1A resulted in an increased MIC of amoxicillin that was almost identical to that for the original Hardenberg strain.**

*Helicobacter pylori*, a spiral-shaped gram-negative bacterium which colonizes the human stomach, is the causative agent of chronic active gastritis and peptic ulcer disease and is associated with increased risk for gastric cancer and gastric lymphoma (3, 9, 21). Anti-*H. pylori* therapy often consists of the  $\beta$ -lactam antibiotic amoxicillin in combination with one or more antimicrobial drugs, a bismuth component, and/or a proton pump inhibitor (9, 12). *H. pylori* is usually sensitive to amoxicillin, but occasionally strains for which an increased MIC is observed have been reported. The first reported amoxicillin-resistant ( $\text{Amx}^r$ ) strains (MIC > 256 mg/liter) were isolated from dyspeptic patients in Italy and the United States (10), but they all rapidly lost their amoxicillin resistance in vitro (8). Subsequently the isolation of a stable amoxicillin-resistant clinical *H. pylori* isolate, the Hardenberg strain, from an 82-year-old Dutch dyspeptic patient was reported (27).

Bacterial resistance against  $\beta$ -lactam antibiotics results mostly from either the production of  $\beta$ -lactamase (16), structural alterations in one of the penicillin-binding proteins (PBPs) (24), or changes in other proteins involved in cell wall synthesis (4, 17, 29). Initial studies with *H. pylori* indicated the presence of three high-molecular-weight PBPs, designated PBP1A, PBP2, and PBP3 (5). Later a fourth, low-molecular-weight PBP, PBP4, was identified (14), and subsequently five other potential PBPs (13) and an *H. pylori*-specific  $\beta$ -lactamase (19) have been described.

Amoxicillin resistance in *H. pylori* was found not to rely on the acquisition or expression of  $\beta$ -lactamase (8, 27). The nonstable amoxicillin resistance of *H. pylori* (8) is probably due to a decreased expression of the penicillin-binding protein PBP1A (7). In the recently described in vitro-selected  $\text{Amx}^r$  *H. pylori* strains, amoxicillin resistance was suggested to result from alterations in PBP1A (6, 22).

In this study, we describe the molecular mechanism of amoxicillin resistance of the Hardenberg strain. Ten genes from the published *H. pylori* genomes were selected as potential candidates based on their putative role in cell wall synthesis (Table 1). After genetic transformation of the amoxicillin-sensitive ( $\text{Amx}^s$ ) strain 1061 with PCR products of the selected candidate genes of the  $\text{Amx}^r$  Hardenberg strain, the minimal PCR fragment able to transfer amoxicillin resistance was sequenced and the amino acid alterations responsible for amoxicillin resistance were identified.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *H. pylori* strains used in this study were the  $\text{Amx}^r$  Hardenberg strain (27) and the  $\text{Amx}^s$  *H. pylori* strains 1061 (11), J99 (2), 26695 (25), and SS1 (15). Bacteria were routinely grown on Columbia agar plates (Becton Dickinson, Cockeysville, Md.) supplemented with 7% lysed horse blood (BioTrading, Mijdrecht, The Netherlands) and *H. pylori*-selective supplement (Oxoid, Basingstoke, United Kingdom) for 48 to 72 h at 37°C in an atmosphere of 5% O<sub>2</sub>, 10% CO<sub>2</sub>, and 85% N<sub>2</sub>. *Escherichia coli* strain DH5 $\alpha$  MCR (Life Technologies BV, Paisley, United Kingdom) was grown on Luria-Bertani agar plates (23) for 24 h at 37°C in an aerobic environment. Selection of *E. coli* DH5 $\alpha$  MCR transformed with pGEM-T Easy clones was performed on ampicillin-containing (100 mg/liter) Luria-Bertani agar plates.

**Determination of MIC.** The MIC was routinely determined by E-test (AB Biodisk, Solna, Sweden) (10) or by agar dilution (8).

**DNA manipulation.** Recombinant DNA techniques were performed according to standard protocols (23). PCR fragments and pGEM-T Easy clones were sequenced on an Amersham Vistra 725 DNA sequencer using Thermo-Seque-

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TABLE 1. Transformation with PCR products of putative Amx<sup>r</sup> genes

| Putative function of selected gene (gene name) <sup>a</sup> | Gene no. <sup>b</sup> | Position of fragment <sup>c</sup> | Transformation frequency <sup>d</sup> |
|---|-----------------------|-----------------------------------|---------------------------------------|
| Penicillin-binding protein ( <i>pbp4</i> )                  | HP0160                | 168848–169882                     | None                                  |
| <i>H. pylori</i> cysteine-rich protein A ( <i>hcpA</i> )    | HP0211                | 218980–218291                     | None                                  |
| Lysis tolerance protein ( <i>lytB</i> )                     | HP0400                | 411955–411354                     | None                                  |
| Penicillin-binding protein ( <i>pbp1A</i> )                 | HP0597                | 632742–631000                     | $5 \times 10^{-6}$                    |
| Rod shape-determining protein ( <i>rodA1</i> )              | HP0743                | 798879–797794                     | None                                  |
| Rod shape-determining protein ( <i>mreC</i> )               | HP1372                | 1436921–1436354                   | None                                  |
| Rod shape-determining protein ( <i>mreB</i> )               | HP1373                | 1438015–1437011                   | None                                  |
| Cell division protein ( <i>fisI</i> )                       | HP1556                | 1639818–1638044                   | None                                  |
| Penicillin-binding protein ( <i>pbp2</i> )                  | HP1565                | 1648924–1647271                   | None                                  |
| Methicillin resistance protein ( <i>llm</i> )               | HP1581                | 1660483–1659552                   | None                                  |

<sup>a</sup> Genes were selected from the published *H. pylori* genomes (2, 25) as potential candidates based on their putative role in cell wall synthesis.

<sup>b</sup> HP gene numbers corresponding to the *H. pylori* 26695 genome sequence (25).

<sup>c</sup> Position of duplicated fragment, corresponding to the *H. pylori* 26695 genome sequence (25). Primers used for the amplification of the PCR fragments started at the outside of the fragment and had each a length of 20 bp.

<sup>d</sup> Determined as the number of Amx<sup>r</sup> colonies per microgram of DNA per recipient CFU. Data represent the means of three experiments.

nase premixed cycle sequencing kit (Amersham, Buckinghamshire, United Kingdom).

**Natural transformation of *H. pylori*.** Bacteria were transformed with approximately 1 µg of total DNA or 200 to 500 ng of PCR product essentially as described by Wang et al. (28). Transformants were selected on Dent plates containing 0.2 to 2 mg of amoxicillin (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) per liter. As a control, bacteria were transformed with Tris-EDTA (TE) (1 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) or DNA from the Amx<sup>s</sup> strain 1061.

**PCR.** The oligonucleotide primers (Isogen, Maarsen, The Netherlands) used in this study are indicated in Table 1 and Fig. 1. PCR was performed in an automated thermal cycler (GeneAmp PCR system 9700; Perkin-Elmer), using the PCR-core system I (Promega, Madison, Wis.).

**Site-directed mutagenesis.** Four different 135-bp oligonucleotides (Isogen) were designed based on the sequence of *pbp1A* of *H. pylori* strain 1061, except that they contained mismatches resulting in none, one, or two amino acid substitutions (Glu<sub>406</sub>→Ala and/or Ser<sub>414</sub>→Arg). For amplification, 5 pmol of the 135-bp oligonucleotides was used as template, with 25 pmol of each terminal

primer (5'-GCTATTCCACGACTTCTAAA-3' and 5'-GCAAGGTTACAAGCCTAAA-3').

**Nucleotide sequence accession numbers.** The *pbp1A* sequences of the *H. pylori* Hardenberg and 1061 strains have been deposited into the GenBank database under accession no. AF479617 and AF479618, respectively.

## RESULTS AND DISCUSSION

**Determination of the MICs of various antibiotics.** The MICs of the four antibiotics commonly used in anti-*H. pylori* therapy, as well as several β-lactam antibiotics, are presented in Table 2. For the Hardenberg strain, the MIC of amoxicillin was 8 mg/liter, while the MIC for strain 1061 was well below 0.016 mg/liter. The MICs of the other β-lactam antibiotics were significantly higher for the Hardenberg strain than for *H.*

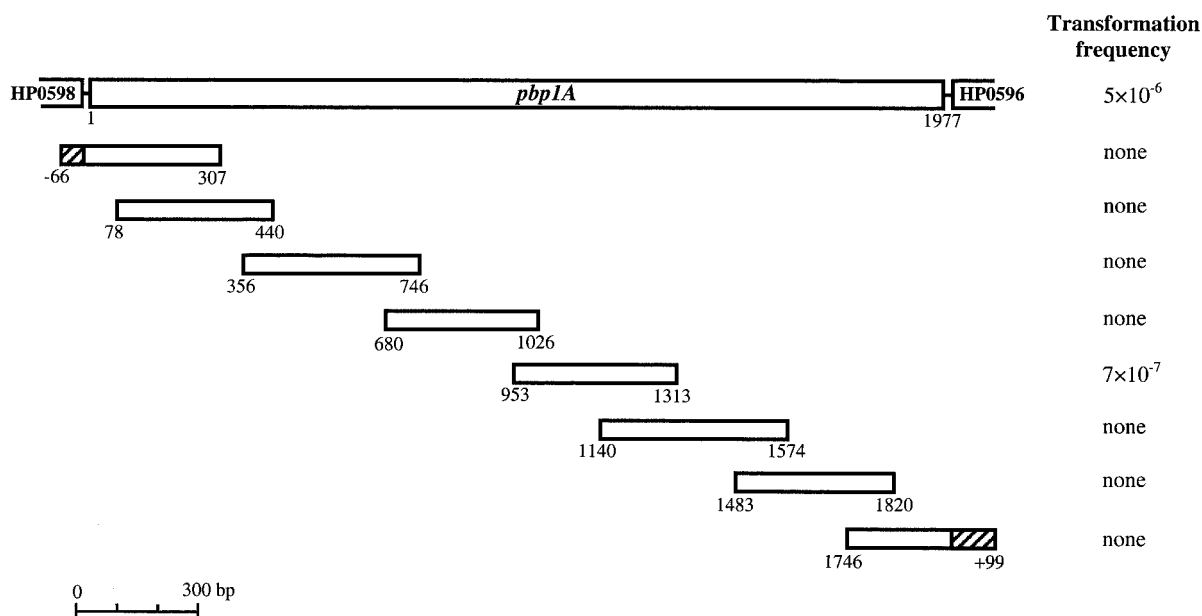


FIG. 1. Identification of the region of *pbp1A* required for amoxicillin resistance. Transformants were obtained by transformation of overlapping PCR fragments of the Amx<sup>r</sup> Hardenberg strain to the Amx<sup>s</sup> strain 1061. The transformation frequency was determined as the number of Amx<sup>r</sup> colonies per microgram of DNA per recipient CFU. Data represent the means of three experiments. Primers used for the amplification of the smaller overlapping PCR fragments started at the outside of the fragment and had each a length of 20 bp. The first and last fragments (hatched areas) partially consist of the flanking genes of *pbp1A*, namely, HP0596 and HP0598.

TABLE 2. MICs for the Hardenberg strain, *H. pylori* strain 1061, and Amx<sup>r</sup> 1061 transformants determined by E-test

| Antibiotic              | MIC (mg/liter) <sup>a</sup> |        |  |
|-------------------------|-----------------------------|--------|--|
|                         | Hardenberg                  | 1061   | Amx <sup>r</sup> 1061 transformants <sup>b</sup> |
| Amoxicillin             | 8                           | <0.016 | 0.75   |
| Amoxicillin-clavulanate | 2                           | <0.016 | 0.25   |
| Cefotaxime              | 0.75                        | 0.023  | 0.75   |
| Ceftazidime             | 4                           | 0.25   | 3  |
| Penicillin              | 2                           | 0.032  | 0.25   |
| Piperacillin            | 4                           | <0.016 | 0.38   |
| Clarithromycin          | <0.016                      | <0.016 | <0.016   |
| Metronidazole           | 0.023                       | 192    | 192  |
| Tetracycline            | <0.016                      | 0.023  | 0.032  |

<sup>a</sup> Data shown are the averages of three separate experiments.

<sup>b</sup> MICs are the averages of 10 randomly selected transformants (obtained after transformation with total DNA of the Hardenberg strain) derived from three independent transformation experiments.

*pylori* strain 1061. MICs of clarithromycin and tetracycline were identical for both strains, but the Hardenberg strain was found to be sensitive to metronidazole, while strain 1061 is known to be resistant to that drug (11).

As the E-test was reported to generate discrepant results when compared to the agar dilution method (1, 20, 26), and as the E-test does not allow determination of amoxicillin MICs below 0.016 mg/liter, the susceptibility to amoxicillin was also tested by agar dilution. The MIC of amoxicillin for the Hardenberg strain by agar dilution was slightly higher (10 mg/liter) than by E-test. For *H. pylori* strain 1061, the MIC of amoxicillin was 0.01 mg/liter.

**Transfer of amoxicillin resistance by natural transformation.** Transformation of *H. pylori* strain 1061 with total DNA of the Hardenberg strain resulted in Amx<sup>r</sup> colonies at a frequency of 10<sup>-5</sup>. The MICs of amoxicillin for 10 randomly selected transformants (obtained from three independent transformation experiments) as determined by E-test (Table 2) and agar dilution were 0.75 and 1.0 mg/liter, respectively. In the agar dilution method, for these transformants the MIC of amoxicillin was 100-fold higher than that for the 1061 acceptor strain, and it was only slightly lower than that of the Hardenberg donor strain. For the Amx<sup>r</sup> transformants, the MICs of the other  $\beta$ -lactam antibiotics also displayed increases (Table 2).

**Transformation with PCR products of cell wall synthesis-encoding genes.** Based on their putative role in cell wall synthesis, 10 genes from the published *H. pylori* genome sequences of strain 26695 (25) and strain J99 (2) were selected as potential Amx<sup>r</sup> genes (Table 1). Amx<sup>s</sup> strain 1061 was transformed with PCR-amplified products of the selected genes of the Amx<sup>r</sup> Hardenberg strain. Only transformation with *pbp1A* resulted in Amx<sup>r</sup> colonies with a frequency of 5  $\times$  10<sup>-6</sup> (Table 1). PCR products of the other putative amoxicillin resistance genes, TE, and DNA from strain 1061 were each unable to transfer amoxicillin resistance. The MIC of amoxicillin for 10 randomly selected Amx<sup>r</sup> colonies (obtained from three independent transformation experiments) was identical to that of strain 1061 transformed with total DNA of the Hardenberg strain. Repeated transformation of these transformants with either the total DNA or the other nine PCR products of the Hardenberg strain did not result in an increase of the MIC (data not

shown). The MIC for these transformants could be increased to the same level as the Hardenberg strain only by continued exposure to amoxicillin, but this additional increase of the resistance was not stable. This indicated that the additional increase in the MIC of amoxicillin for the Amx<sup>r</sup> 1061 transformants was due to a transient physiological change. Apparently a stable increase of amoxicillin resistance to the level of the Hardenberg strain requires mutations in more than one locus, and the likelihood that these are all transferred by transformation is relatively small.

**Identification of *pbp1A* mutations involved in amoxicillin resistance.** To determine the mutations of *pbp1A* responsible for amoxicillin resistance, natural transformation experiments were performed by using small overlapping PCR fragments of *pbp1A* (Fig. 1). Amx<sup>r</sup> 1061 transformants (7  $\times$  10<sup>-7</sup>) were observed only with the 361-bp DNA fragment that spanned nucleotides 953 to 1313 (numbering according to *pbp1A* of *H. pylori* strain 26695). No Amx<sup>r</sup> colonies were observed after transformation with the seven other *pbp1A* fragments, TE, or DNA from strain 1061. The MIC of amoxicillin for 10 randomly selected transformants (obtained from three independent transformation experiments) was 1.0 mg/liter.

Analysis of PBP1A sequences obtained from the Hardenberg strain, the Amx<sup>s</sup> 1061 strain, and four Amx<sup>r</sup> 1061 transformants (obtained from three independent transformation experiments with total DNA from the Hardenberg strain) showed several amino acid substitutions in the Hardenberg strain as well as in the transformants that did not occur in the Amx<sup>s</sup> strain. Three transformants had incorporated the complete Hardenberg *pbp1A* sequence, while the fourth transformant contained only the second half of the gene. For this fourth transformant the DNA exchange apparently occurred between nucleotides 793 and 854. This was independent proof that the first 792 bp of *pbp1A* of the Hardenberg strain are not involved in amoxicillin resistance, as was already indicated by transformation with the *pbp1A* PCR fragments.

**Amoxicillin resistance of *pbp1A* is due to the Ser<sub>414</sub>→Arg substitution.** Sequence analysis of PBP1A showed two amino acid differences in the region between nucleotides 953 and 1313, a glutamic acid by an alanine (Glu<sub>406</sub>→Ala) and a serine by an arginine (Ser<sub>414</sub>→Arg). In order to identify which of these amino acid changes was responsible for the increase in amoxicillin resistance, site-directed mutagenesis was used. Strain 1061 was transformed with four different 135-bp fragments coding for amino acids 381 to 426 (numbering according to *pbp1A* of *H. pylori* strain 26695 [25]) containing either no, one (Glu<sub>406</sub>→Ala or Ser<sub>414</sub>→Arg), or both substitutions. Amx<sup>r</sup> colonies were observed only after natural transformation with the two fragments that contained the Ser<sub>414</sub>→Arg substitution with transformation frequencies of 7  $\times$  10<sup>-8</sup> and 4  $\times$  10<sup>-8</sup> respectively. Sequence analysis of PBP1A of four randomly selected transformants, obtained from two independent transformation experiments with the 135-bp Ser<sub>414</sub>→Arg fragment, confirmed that only this substitution was present in the Amx<sup>r</sup> transformants. The MIC of amoxicillin for these transformants was 1.0 mg/liter. In addition to the MICs of amoxicillin for these organisms, the growth rates (optical density at 600 nm measured at regular intervals during a time period of 48 h), sizes, and shapes of the bacteria were determined. In the ab-

sence of amoxicillin, no differences between the Hardenberg strain, the four Amx<sup>r</sup> transformants, and strain 1061 were found in growth rates, sizes, or shapes of the bacteria. Under amoxicillin pressure (1 mg/liter), the bacteria became a little bit shorter and thicker, but in spite of this morphological change there was no significant change in growth rates (data not shown).

Site-directed mutagenesis indicated that the Ser<sub>414</sub>→Arg substitution in PBP1A represents the main factor in amoxicillin resistance of the Hardenberg strain. Recently, other investigators (22) reported that amino acid substitutions Tyr<sub>484</sub>→Cys, Thr<sub>541</sub>→Ile, and Pro<sub>600</sub>→Thr in PBP1A are responsible for amoxicillin resistance of an in vitro-selected Amx<sup>r</sup> *H. pylori* strain. None of these amino acid substitutions play a role in amoxicillin resistance of the Hardenberg strain, since these substitutions were not present in the Amx<sup>r</sup> Hardenberg strain. Further, the Ser<sub>414</sub>→Arg substitution in PBP1A in the Hardenberg strain and Amx<sup>r</sup> 1061 transformants had no influence on the expressed amount of PBP1A molecules compared to the wild-type PBP1A of *H. pylori* strain 1061 (data not shown). Probably the amino acid substitutions in PBP1A of the Hardenberg strain and the in vitro-selected Amx<sup>r</sup> strain (22) affect the binding affinity of PBP1A for penicillins, but this was not tested.

**Role of *pbp1A* in amoxicillin resistance of other *H. pylori* strains.** To test the generality of our findings, we also transformed *H. pylori* strains J99, 26695, and SS1 (MICs of amoxicillin for these strains, 0.01, 0.01, and 0.2 mg/liter, respectively) either with the PCR product of *pbp1A* or with total DNA from the Hardenberg strain. With all strains, Amx<sup>r</sup> transformants were isolated at high frequency. Ten Amx<sup>r</sup> transformants of each strain (obtained from two independent transformation experiments) were selected at random, and the MICs of amoxicillin were determined. All transformants had a MIC of 1.0 mg/liter, which was identical to the MIC of the Amx<sup>r</sup> *H. pylori* 1061 transformants but lower than the MIC of the Amx<sup>r</sup> Hardenberg strain. These results indicated that for strains other than 1061, the introduction of the *pbp1A* from the Hardenberg strain also increased the MIC of amoxicillin.

The results of our study showed that a single serine-to-arginine substitution in PBP1A induces high-level amoxicillin resistance in *H. pylori*. Since the existence of *H. pylori* strains with a moderate resistance level against amoxicillin already has been reported (18, 30) and apparently a single mutation is sufficient to increase the MIC of amoxicillin, an increase of naturally occurring *H. pylori* strains with a high level of amoxicillin resistance can be expected in the near future.

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