Identification of a Novel Penicillin-Binding Protein from *Helicobacter pylori*

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The *Helicobacter pylori* genome encodes four penicillin-binding proteins (PBPs). PBPs 1, 2, and 3 exhibit similarities to known PBPs. The sequence of PBP 4 is unique in that it displays a novel combination of two highly conserved PBP motifs and an absence of a third motif. Expression of PBP 4, but not PBP 1, 2, or 3, is significantly increased during mid- to late-log-phase growth.

Helicobacter pylori is a spiral, gram-negative bacterium which causes chronic superficial gastritis and is associated with peptic ulcer disease, gastric carcinoma, and gastric lymphoma (2, 19, 22–24). *H. pylori* appears to be unique in that a number of typical cytoplasmic proteins, including urease, heat shock proteins A and B (HspA and HspB), catalase, and superoxide dismutase, are associated with the outer membrane (4, 6–8, 14, 26, 32). Surface-adsorbed urease (most likely derived from autolyzed bacteria) appears to be essential for survival of *H. pylori* at pH 2, a condition routinely encountered by the bacterium in the human stomach (7, 16, 26).

Autolytic mechanisms in bacteria presumably involve disruption of the peptidoglycan (PG) layer, since it is the backbone of the cell structure. The enzymes capable of degradation and synthesis of PG can be classified into two broad classes, penicillin-binding proteins (PBPs) and non-PBPs (11, 21). In general, PBPs capable of degrading PG exhibit endopeptidase and/or carboxypeptidase activity (33, 34). In this communication, we describe a novel *H. pylori* PBP with unique properties.

To identify *H. pylori* PBPs, fluorescein-C₆-aminopenicillanic acid (Flu-6-APA) was prepared and used to covalently label permeabilized bacteria grown on agar plates (10, 26, 29). Protein preparations normalized for total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5, 18, 30). For detection of Flu-6-APA-labeled proteins, gels were scanned in a Fluorimager. In *H. pylori* 84-183, four PBPs, ranging in molecular mass from 32 to 66 kDa, were identified. Three high-molecular-weight PBPs were in the size range (55 to 66 kDa) of PBPs A, B, and C, described by Ikeda et al. (15) (Fig. 1a, lane 1). We refer to PBPs A, B, and C as PBPs 1, 2, and 3, respectively, since in general PBPs are labeled numerically rather than alphabetically (34). A fourth low-molecular-weight PBP, of approximately 32 kDa (PBP 4), not identified by Ikeda and colleagues was observed consistently in our preparations (Fig. 1a, lane 1). As a negative control to confirm that PBPs in whole-cell extracts bind to the penicillin moiety of Flu-6-APA and not to the fluorescein moiety, whole-cell extracts were incubated with fluorescein-di-

galactose. Neutral hydroxylamine specifically removes penicillin (and thereby fluorescein) from PBPs (3). Disappearance of PBPs after treatment with neutral hydroxylamine was used as an additional control. No protein bands were visible with fluorescein-di-galactose labeling, indicating that there is no non-specific binding of proteins to the fluorescein label (Fig. 1a, lanes 3 and 4). Similarly, no protein bands were visible after treatment of Flu-6-APA-treated protein samples with neutral hydroxylamine (Fig. 1a, lane 2), further confirming the specificity of PBP binding to Flu-6-APA. The fluorescence intensity of PBP 4 was consistently greater than that of the other three PBPs, most likely due to increased expression of PBP 4 compared to the other three PBPs (see below). Pretreatment of whole cells with EDTA did not inhibit the ability of PBP 4 to bind to Flu-6-APA, indicating (data not shown) that metal ions are not required for penicillin binding; hence, PBP 4 is unlikely to be related to metallo- β -lactamases (25).

During characterization of PBP 4, we noticed significant loss of PBP 4 signal in membrane preparations compared with whole cells. Therefore, we investigated the possibility that PBP 4 might be distributed in both the membrane and the soluble (nonmembrane) fractions due to mechanical separation of loosely bound PBP 4 from the bacterial membrane fraction during centrifugation. *H. pylori* 84-183 membrane and soluble fractions were prepared (28) after harvesting of bacteria from agar plates into phosphate-buffered saline. Figure 1b shows that unlike most PBPs (11–13), PBP 4 of *H. pylori* is present both in membrane fractions and in soluble fractions. By analogy with *E. coli* PBP 4, it is likely that a fraction of *H. pylori* PBP 4 is loosely associated with the membrane and that this association is disrupted during biochemical preparation of the membrane and soluble fractions (17). In contrast, the high-molecular-weight PBPs of *H. pylori* were observed exclusively in membrane preparations (Fig. 1b). There is no apparent difference between the molecular weights of PBP 4 present in these two fractions.

We investigated the possibility that PBP 4 expression might vary with the growth phase, since PBP 4 was not detected in very early log phase cultures (12 h) of *H. pylori* by Ikeda et al. (15). For this purpose, *H. pylori* 84-183 early-log-phase (24-h), mid-log-phase (48-h), late-log-phase (72-h), and extremely late-log-phase (96-h) cultures were harvested (16) and whole cells were labeled with Flu-6-APA. As seen in Fig. 2, a distinct difference in the total amounts of PBP 4 present in a fresh

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FIG. 1. (a) PBP profile of *H. pylori*. Flu-6-APA-labeled *H. pylori* whole-cell extracts were separated by SDS-12% PAGE; the molecular mass standards are indicated in kilodaltons. Lane C, no Flu-6-APA added (negative control); lane 1, *H. pylori* 84-183 labeled with 10 μ M (final concentration) Flu-6-APA; lane 2, *H. pylori* 84-183 labeled with 10 μ M (final concentration) Flu-6-APA and then treated with hydroxylamine (hydroxylamine treatment disrupts the covalent linkage with PBPs, resulting in release of the label); lane 3, *H. pylori* 84-183 labeled with 10 mM fluorescein-di-galactose; lane 4, *H. pylori* 84-183 labeled with 100 mM fluorescein-di-galactose. PBPs 1, 2, and 3, seen in lane 1, presumably represent PBPs A, B, and C, respectively, as reported by Ikeda et al. (15). (b) Distribution of PBP 4 between membrane and soluble fractions. *H. pylori* 84-183 membrane and soluble fractions normalized for total protein content were labeled with Flu-6-APA, and equal amounts of total proteins were analyzed by SDS-12% PAGE. Lane 1, *E. coli* K-12 strain MC1061 whole cells (16); lane 2, *H. pylori* 84-183 whole-cell extracts; lane 3, *H. pylori* soluble fraction; lane 4, *H. pylori* membrane fraction.

subculture and a repeated subculture is noticeable. In freshly subcultured bacteria, the amount of PBP 4 is low in early-log-phase cultures and increases in mid- to late-log-phase cultures (Fig. 2a). In contrast, in repeatedly subcultured *H. pylori* 84-183, expression levels of PBP 4 are similar from early through late log phase while decreasing in extreme late log phase (Fig. 2b). Expression levels of the three high-molecular-weight PBPs, however, are similar in freshly and repeatedly subcultured *H. pylori* at all growth stages (Fig. 2).

H. pylori PBP 4 was detected in all strains analyzed. The size of PBP 4 varied from 30 to 36 kDa between different strains, as seen in Fig. 3. There was also some variation in the profile of high-molecular-weight PBPs. The significance of this variation is currently unknown but is consistent with the significant genetic variability present in *H. pylori* genes (9). In all strains tested, the amount of fluorescence associated with PBP 4 was relatively greater than that of the high-molecular-weight PBPs.

H. pylori PBP 4 was purified on a penicillin affinity column (3). The PBPs eluted from the affinity column were separated by gel electrophoresis. Based on silver staining of the resultant gel, the amount of PBP 4 purified from the affinity column was

FIG. 2. Time course of PBP 4 expression. (a) *H. pylori* 84-183 (fresh subculture) whole cells harvested at early (24-h), mid (48-h), late (72-h), and extremely late (96-h) log phase were normalized for total protein content and labeled with Flu-6-APA, and an equal amount of total protein was analyzed by SDS-12% PAGE. Lane C, no Flu-6-APA added; lane F1, early-log-phase (24-h) culture; lane F2, mid-log-phase (48-h) culture; lane F3, late-log-phase (72-h) culture; lane F4, extremely late-log-phase (96-h) culture. (b) *H. pylori* 84-183 (repeat subculture) whole cells harvested at early (24-h), mid (48-h), late (72-h), and extremely late (96-h) log phase were normalized for total protein content and labeled with Flu-6-APA, and an equal amount of total protein was analyzed by SDS-12% PAGE. Lane C, no Flu-6-APA added; lane R1, early-log-phase (24-h) culture; lane R2, mid-log-phase (48-h) culture; lane R3, late-log-phase (72-h) culture; lane R4, extremely late-log-phase (96-h) culture.

significantly greater than that of the three high-molecular-weight PBPs, suggesting that quantitatively more PBP 4 is present in *H. pylori* (data not shown). The low-molecular-weight PBP 4 was subjected to N-terminal sequencing (20, 30). A single N-terminal sequence, ??GEKYFKMANQALK, was obtained from both membrane and soluble fractions. The first two residues of PBP 4 could not be identified by N-terminal sequencing.

The PBP 4 protein is encoded by open reading frame (ORF) HP0160 and JHP148 according to the TIGR and ASTRA *H. pylori* genome sequence databases, respectively (Fig. 4) (1, 35). The PBP 4 gene encodes a 306-amino-acid protein (Fig. 4) that is rich in cysteine (4.5%) and has a predicted molecular mass of 34 kDa (ORF HP0160 [35]). The predicted molecular mass of the mature form of PBP 4 (25-amino-acid signal sequence processed form) is 31,189 Da. There are two amino acid substitutions in the N-terminal amino acid sequences of PBP 4 between *H. pylori* 84-183 and 26695. The predicted N-terminal amino acid sequence of PBP 4 from *H. pylori* J99 is identical to the N-terminal amino acid sequence of strain 84-183, as reported above (1). The sequence of PBP 4 shows no homology to any known PBP or proteins from other bacteria.

There are three possible explanations for the lack of detection of PBP 4 by Ikeda et al. (15). First, they used 14 C-labeled penicillin to detect PBPs whereas we used Flu-6-APA labeling

FIG. 3. Size variation of PBP 4 in different strains. Whole cells from different *H. pylori* strains were normalized for total protein content and labeled with Flu-6-APA, and an equal amount of total protein was analyzed by SDS-12% PAGE. Lane C, no Flu-6-APA added; lane 1, strain CK44; lane 2, strain HH12; lane 3, strain RJ17; lane 4, strain GS; lane 5, strain N6; lane 6, strain WV8D3; lane 7, strain A5; lane 8, strain HM; lane 9, strain 84-183. Lanes 1 to 4 and lanes 6 and 8 are fresh clinical strains which were obtained from human subjects undergoing endoscopy for dyspeptic symptoms and have been minimally passaged in the laboratory. Lanes 5, 7, and 9 are laboratory strains which, although initially isolated from humans, have been extensively passaged in the laboratory (16).

to analyze PBPs. However, since we were able to detect much more silver-stainable PBP 4 in our purification procedures (which do not rely on binding of Flu-6-APA to PBPs) relative to the three high-molecular-weight PBPs, it is unlikely that the method used for labeling is the source of the difference. The second difference between these two studies is that our cultures were routinely grown on blood agar plates, while Ikeda et al. used liquid broth cultures. The third difference between our PBP studies and those of Ikeda et al. is the culture age of the bacterial sample. Ikeda et al. (15) used a very early log phase (12-h) culture for their protein isolations, while we routinely used late-log-phase (72-h) cultures in our assays. Interestingly, expression of PBP 4 appears to be minimal in early-log-phase cultures and maximal in older cultures. However, we cannot exclude the possibility that all of the PBP 4 in the membrane preparations employed by Ikeda et al. (15) was present in a form loosely associated with the cell membrane and was therefore not detected.

Analysis of the genome sequence from *H. pylori* 26695 (35) or J99 (1) indicates that the protein sequence of PBP 4 belongs to a family of seven paralogous genes (on the basis of moderate sequence similarity [$P = 10^{-50}$]) that are scattered throughout

the genome (27). At least one member of this family, HcpA, is expressed in *H. pylori* ATCC 49503 (27). We were unable to detect penicillin binding to any member of this family (except PBP 4) in any of the strains examined. Therefore, the significance of above sequence similarity is unclear. In contrast to the sequence of PBP 4, sequence similarity searches of the *H. pylori* genome have identified three genes (ORFs HP 597, 1556, and 1565 in the TIGR genome database [35] and ORFs JHP 544, 1464, and 1473 in the ASTRA genome database [1]) that show significant homologies to PBPs from other bacteria (13, 34). These three ORFs are likely to correspond to PBPs 1, 2, and 3, as identified by Ikeda et al. (15) and in the present study.

Catalytic centers of PBPs have a remarkably well-conserved topology, and the residues involved in penicillin binding are referred to as motifs: motif 1 (SXXK), motif 2 [S(Y)XN(C)], and motif 3 [K(H,R)T(S)G] comprise the catalytic center (13, 34). In all PBPs identified to date, these three motifs are arranged in a specific order from the N to C termini within PBPs, with motif 1 being the first in the order followed by motif 2 and then motif 3 (13, 34). The sequence of PBP 4 from strain 26695 (35) shows a single conserved SXXK motif. However, the sequence of PBP 4 from strain J99 (1) shows two SXXK motifs, one of which is in the same position as that in strain 26695. We have verified the sequence of the PBP 4 gene in strain 26695 (by direct DNA sequencing of PCR fragments) and have found no differences from the reported sequence (data not shown). The sequences of PBP 4 from both strains show conserved SXN motifs but not the conserved K(H,R)T(S)G motif. Further, in the sequence of PBP 4 the SXXK motif is present near the C terminus following the SXN motif (Fig. 4). The significance of the unique arrangement of PBP motifs in PBP 4 is not known.

Based on the lack of sequence similarity with known PBPs and the unique arrangement of the PBP motifs, PBP 4 may carry out a unique reaction(s) and/or may contribute to the spiral morphology of *H. pylori* (19). When a low-molecular-weight PBP (PBP G) from *Pseudomonas aeruginosa* with extensive homology to *Escherichia coli* D,D-endopeptidase (PBP 4 of *E. coli*) is overproduced in *E. coli*, lysis of cells results (31). It is worth pointing out that *H. pylori* PBP 4 is a low-molecular-weight PBP and appears to be overproduced compared to PBPs 1, 2, and 3. Additional experiments are required to determine the function(s) of PBP 4 in *H. pylori*. At present, we

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MIKSWTKKWFLILFLMASCSSYLVA TTGEKYFKMATQAFKRGDYHKAVAFYKR

SCMLRVGVGCTSLGSMYEDGDGVDQNITKAVFYRRGCNLRNHLACASLGSMY

EDGDGVQKNLPKAIYYRRGCHLKGGVSCGSLGFMYFNGTGVKQNYAKALFLS

KYACSLNYGISCMFVGYMYRNAKGVQKDLKKALANFKRGCHLKDGASCVS LGY

MYEVMGMDVKQNGEQALNLYKKGCYLKRGSGCHNVAVMYYTGKGVPKDLDAI

SYYKKGCTLGFSGCKVLEEVIGKKSDDLQDDAQNDTQDDMQ

FIG. 4. Deduced amino acid sequence of PBP 4 protein (ORF HP0160). The sequence begins with an initiation methionine. The signal peptidase cleavage site is denoted by an arrow, and the signal peptide sequence is underlined. The PBP 4 sequence lacks the conserved KTG motif. However, several regions in the PBP 4 sequence differ from the conserved KTG motif. However, several regions in the PBP 4 sequence differ from the conserved KTG motif by a single residue; these sites are italicized and underlined. Possible SXN motifs are italicized and marked with dotted underlines. The active site of all known PBPs (SXXK) is highlighted (36).

are analyzing the composition of *H. pylori* PG to determine the structure of the possible substrate for PBP 4.

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