Novel Antigens of *Helicobacter pylori* Correspond to Ulcer-Related Antibody Pattern of Sera from Infected Patients

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Recently, we reported that the patterns of antibodies to *Helicobacter pylori* protein antigens in serum may be useful for screening patients at high risk for ulcers (P. Aucher et al., *J. Clin. Microbiol.* 36:931–936, 1998). Here we report the identification, by a combination of electrophoretic, immunochemical, and protein sequencing methods, of five antigens that correspond to this antibody pattern: groEL, catalase A, flagellin A, beta-ketoacyl-acyl carrier protein synthase I (β-ketoacyl-ACP S), and peptidyl prolyl cis-trans isomerase (PPIase). β-Ketoacyl-ACP S and PPIase are reported for the first time as antigens of diagnostic interest in infections by *H. pylori*. The antigenicity of the five antigens, together with those of CagA and VacA, was tested in an immunoblot assay with water-soluble protein extracts from two *H. pylori* pathogenic strains (HP 141 and ATCC 43579) and panels of sera from *H. pylori*-positive patients with gastroduodenal ulcers (GDU), nonulcer dyspepsia (NUD), as well as sera from *H. pylori*-negative healthy volunteers. For catalase A, groEL, and flagellin A antigens, no overall statistically important values were found making it possible to discriminate between patients with GDU and NUD. For both *H. pylori* strains, the mean performance indices (MPI) presenting percentages of correctly classified patients with GDU and NUD showed that the most significant antibody patterns were as follows: anti-VacA + anti-β-ketoacyl-ACP S (MPI = 76.1), anti-VacA + anti-PPIase (MPI = 71.8), and anti-CagA + anti-VacA + anti-β-ketoacyl-ACP S (MPI = 70.5). Antibody patterns detected with these antigen profiles may therefore be useful in developing a diagnostic test designed to predict the clinical severity of the *H. pylori* infection within the adult population of France.

Serological tests and assays for the diagnosis of *Helicobacter pylori* infection are included among the noninvasive methods recommended by the European *Helicobacter pylori* Study Group (7). Evaluation of the humoral immune response to *H. pylori* antigens by immunoblotting is a valuable alternative and complement to the more routinely used enzyme-linked immunosorbent assay (ELISA) tests (8, 13, 14, 19, 25). Immunoblotting appears to be sometimes more sensitive and useful for detecting low-abundance antibodies and antibodies directed toward nonconformational epitopes of immunodominant antigens. By this method, we recently reported that several antigens of *H. pylori* are preferentially recognized by the serum antibodies of adult French patients with gastroduodenal ulcers (GDU) that had been infected by this gastric pathogen. We therefore postulated that the five antigens corresponding to this antibody pattern could be useful for differentiating patients at high risk of GDU from patients with nonulcer dyspepsias (NUD) (2). Under the conditions of an immunoblot assay, two proteins of this antigenic profile were identified as CagA (125 kDa) and VacA (87 kDa), while the three other proteins were assigned only as immunoreactive bands with approximate molecular masses of 54, 42, and 35 kDa (denoted, respectively, p54, p42, and p35). Knowledge of the exact primary structure of p54, p42, and p35 is thus an essential prerequisite for preparing and further analyzing the antigenic preparation designed for the development of a test predicting the clinical outcome of the *H. pylori* infection.

Our initial aim was to purify the three unidentified antigens in order to determine their amino acid sequences. After optimizing the electrophoretic separation of these antigens, we differentiated three antigens within the zone of p54. This prompted us to reinvestigate all members of antigenic profile by an immunoblot assay by using panels of sera from *H. pylori*-positive patients with GDU and NUD, as well as with the control sera of *H. pylori*-negative healthy volunteers.

**MATERIALS AND METHODS**

**Patients and patient sera.** A total of 115 individual sera were collected in the university hospitals of Poitiers (southwest), Nancy (east), and Brest (northwest). These cities represent three geographical locations in France situated at a distance of more than 700 km from one another. Eighty-five sera were obtained from patients attending the departments of hepatology-gastroenterology from 1995 to 2000. All patients underwent upper gastroduodenal endoscopy with multiple antral and fundic biopsies that were processed for histology. These subjects had received neither antimicrobial nor antisecretory therapies during the previous 3 months. As determined by in-house ELISA (29), all patients were seropositive for *H. pylori*. Of the 85 patients, 55 (43 males and 12 females) had GDU, and 30 (17 males and 13 females) showed signs of gastritis or a dyspeptic symptoms but were ulcer-free (NUD). For the GDU patient group, the median ages were 60.8 years (range, 24 to 77 years) and 69.5 years (range, 51 to 93 years) for males and females, respectively; the mean ELISA index was 0.63. Thirty control sera were collected from healthy blood donors (16 males and 14 females). The median ages were 40.8 years (range, 28 to 51 years) and 35.8 years (range, 21 to 46 years) for males and females, respectively. All persons within this control
ATCC 43579 was used in our previous study (2). H. pylori di
duoride (PVDF) Sequi-Blot membranes (Bio-Rad) at a constant current of
dling, storage, and processing of these sera were as described previously (2).
end of electrophoresis, slab gels were brie
y rinsed in Towbin buffer containing
chamber was Tris-glycine (pH 8.3) containing 0.1 mM thioglycolate. After the
–
H. pylori
soluble proteins of the saline extract from both
Antigen Classification (J99/26695) Pairwise protein identity (%) Mol mass (kDa) (J99/26695) Protein length (aa) (J99/26695) Predicted pI (J99/26695) Sequenced fragment (strain HP 141) Identity of HP 141 sequences with J99/26695 (%) N-terminal position on J99/26695
CATALASE A SUBUNIT JHP 809/HP 875 97.4 58.6/58.3 505/505 9.04/8.89 MVNKDV 100/100 1–6
groEL JHP 008/HP 010 99.6 58.26/58.24 546/546 5.48/5.46 AKEIKFS 100/100 2–8
β-Ketoacyl-ACP S JHP 505/HP 558 97.3 43.6/43.56 412/412 6.63/6.52 VEYGYNV 100/100 286–302
PPIase JHP 161/HP 175 97 34.03/34.04 299/299 9.96/10.04 KPAHNANN\textunderbar{TH}NTK\textkappa 100/87 22–36


d corresponds to the second (by importance) most likely cleavage site between positions 21 and 22 (LMA-KP) producing N-terminal signal peptide. Determined by using SignalP predictions based on neural networks and hidden Markov models of gram-negative bacteria (Nielsen et al. [18]; SignalP server [http://www.cbs.dtu.dk/ services/SignalP-2.0]).

ELECTROPHORESIS, IMMUNOBLOT ASSAY, AND PROTEIN MICROSEQUENCING. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 100-µg portions of water-soluble proteins was carried out on 4% stacking–10% resolving gels and 1-mm-thick polyacrylamide gels, with a 16-cm net distance of migration within the resolving gel, by using a Maxi-Gel apparatus (Bio-Rad) under a constant current of 12 mA for 16 h. The electrophoresis buffer in the upper chamber was Tris-glycine (pH 8.3) containing 0.1 mM thioglycolate. After the end of electrophoresis, slab gels were briefly rinsed in Towbin buffer containing 0.1 mM thioglycolate and then subjected to Western transfer onto polyvinylidene difluoride (PVDF) Sequi-Blot membranes (Bio-Rad) at a constant current of 1.56 mA/cm² of membrane for 1 h. For controlling transfer quality, some PVDF strips from each Western blot were processed with different stains (i.e., Amido-black 10B or CBB); the remaining PVDF strips were used in an immunoblot assay. The resulting N-terminal or internal sequences were compared to

<table>
<thead>
<tr>
<th>Protein Subunit</th>
<th>Predicted pI</th>
<th>Sequence (strain HP 141)</th>
<th>Identity of HP 141 sequences with J99/26695 (%)</th>
<th>N-terminal position on J99/26695</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATALASE A SUBUNIT</td>
<td>9.04/8.89</td>
<td>MVNKDV</td>
<td>100/100</td>
<td>1–6</td>
</tr>
<tr>
<td>groEL</td>
<td>5.48/5.46</td>
<td>AKEIKFS</td>
<td>100/100</td>
<td>2–8</td>
</tr>
<tr>
<td>β-Ketoacyl-ACP S</td>
<td>6.63/6.52</td>
<td>VEYGYNV</td>
<td>100/100</td>
<td>286–302</td>
</tr>
<tr>
<td>PPIase</td>
<td>9.96/10.04</td>
<td>KPAHNANN\textunderbar{TH}NTK\textkappa</td>
<td>100/87</td>
<td>22–36</td>
</tr>
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</table>

Identify the protein antigens. In an initial experiment, the electrophoretically separated proteins of both strains HP 141 and ATCC 43579 displayed very similar band profiles within the zones of interest encompassing silver-stained p54, p42, and p35. However, the protein bands of HP 141 extract within the zone of p54 were better differentiated than those of ATCC 43579 (data not shown). Strain HP 141 was therefore used as the sole source for further protein purification and sequence analysis. We next optimized the electrophoretic separations of proteins in terms of migration time and protein concentrations to be loaded, conditions of Western transfer, and immunolocalization of protein bands to be sequenced. Four proteins were successfully sequenced by N-terminal Edman degradation: three proteins within the zone of p54 and one protein within the zone of p35. The antigen protein within the zone of p42 turned out to be blocked at its N terminus, and its sequence was determined by internal microsequencing.

As presented in Table 1, comparisons of determined sequences of HP 141 strain with those of both H. pylori strains with known complete genomes (26695 and J99) made it possible to identify five antigenic proteins, namely, catase A subunit (catase A), groEL (other common or recent denominations of this protein are HspB and DSP60), flagellin A subunit (flagellin A), beta-ketoacyl-acyl carrier protein syn-
the CagA and VacA antigens of HP 141 and ATCC 43579 (as determined by chi-square test; \( P \leq 0.05 \)).

\( ^a \) That is, the percentage of patients with ulcers that are correctly classified (i.e., The number of true positive plus the number of negative immunoblot controls/the total number), where the total number is 115.

\( ^b \) MPI for both \( H. pylori \) strains.

\( ^c \) Antibody specificities more frequent in patients with GDU than in patients with NUD concern antigens VacA and catalase A of strain HP 141 and \( \beta \)-ketoacyl-ACP S and PPiase of both \( H. pylori \) strains.
programmed bacterial autolysis that occurs in vitro (22), as well in vivo (6). The latter means of membrane expression has also been suggested for catalase (22), and there is evidence that \textit{H. pylori} catalase may be located both throughout the cytosol and in the periplasmic space (9). In this regard, it is not excluded that, due to unique characteristics of the outer membrane of \textit{H. pylori}, other cytoplasmic proteins may become absorbed. It is also possible that \textit{H. pylori} possesses an as-yet-unknown mechanism for exporting to the cell surface enzymes and other molecules that are important for colonizing the stomach.

As far as we know, CagA, VacA, and \textit{\beta}-ketoacyl-ACP S are reported here for the first time as \textit{H. pylori} antigens of clinical relevance. Because of the lack of information about CagA, VacA, and \textit{\beta}-ketoacyl-ACP S in \textit{H. pylori}, we briefly mention here some aspects of the homologs to these two molecules in other bacterial species. In our opinion, these findings give an idea about the biological importance of these molecules and as

FIG. 1. Factorial analysis of the correspondence of the antibody pattern defined by the presence of antibodies to CagA, VacA, and \textit{\beta}-ketoacyl-ACP S by using \textit{H. pylori} HP 141. The different areas of the circles are proportional to the number of sera of each clinical category: clear areas show the projection of 30 seronegative healthy volunteers, open areas with black dots show the projection of 30 patients with NUD, and dark areas with white dots show the projection of 55 patients with GDU. With the exception of one case, all of the GDU patients are situated on the right side of the diagonal line drawn, and they are mainly gathered in a single group. The healthy volunteers are gathered on the left. The NUD are interspersed over the factorial analysis plane.

<table>
<thead>
<tr>
<th>Antibody pattern to antigen combination (i.e., &quot;profile&quot;)</th>
<th>No. of patterns (%)</th>
<th>Patients with GDU (n = 55)</th>
<th>Patients with NUD (n = 30)</th>
<th>Healthy volunteers (n = 30)</th>
<th>Performance index (%) (n = 115)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CagA + VacA + PPhase</td>
<td>27 (49.0)</td>
<td>21 (38.2)</td>
<td>4 (13.3)</td>
<td>4 (13.3)</td>
<td>72.2</td>
</tr>
<tr>
<td>CagA + VacA + \textit{\beta}-ketoacyl-ACP S</td>
<td>37 (67.3)</td>
<td>21 (38.2)</td>
<td>10 (33.3)</td>
<td>6 (20.0)</td>
<td>75.7</td>
</tr>
<tr>
<td>VacA + PPhase</td>
<td>30 (54.5)</td>
<td>24 (43.6)</td>
<td>4 (13.3)</td>
<td>5 (16.7)</td>
<td>74.8</td>
</tr>
<tr>
<td>VacA + \textit{\beta}-ketoacyl-ACP S + PPhase</td>
<td>27 (49.0)</td>
<td>14 (25.4)</td>
<td>2 (6.7)</td>
<td>4 (13.3)</td>
<td>73.9</td>
</tr>
<tr>
<td>VacA + \textit{\beta}-ketoacyl-ACP S</td>
<td>45 (81.8)</td>
<td>28 (50.9)</td>
<td>11 (36.6)</td>
<td>7 (23.3)</td>
<td>81.7</td>
</tr>
<tr>
<td>CagA + \textit{\beta}-ketoacyl-ACP S + PPhase</td>
<td>28 (51.0)</td>
<td>18 (32.7)</td>
<td>4 (13.3)</td>
<td>2 (6.6)</td>
<td>73.0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The antibody patterns for all antigen profiles were significantly more frequent in patients with GDU than in patients with NUD, except for the antigen combinations CagA + VacA + \textit{\beta}-ketoacyl-ACP S and VacA + \textit{\beta}-ketoacyl-ACPS + PPiase of strain ATCC 43579 (as determined by chi-square test; \( P \leq 0.05 \)).

\textsuperscript{b} That is, the percentage of patients with ulcers that are correctly classified (i.e., the number of true positive + the number of negative immunoblot controls/the total number), where the total number is 115.

\textsuperscript{c} MPI for both \textit{H. pylori} strains.
potential candidates in developing drug, vaccine, and diagnostic assays. β-Ketoacyl-ACP S of H. pylori (gene fabB) belongs to the family of β-ketoacyl synthases, key enzymes involved in the biosynthesis of fatty acids and polyketides. At present, β-ketoacyl-ACP S from E. coli is being studied structurally as a promising drug target for novel antibiotics to treat tuberculosis, as well as for inhibiting certain cancers (16, 23). Antigenic PPIase from H. pylori HP 141 is evolutionarily related to the cyclophilins, FKBP5 (proteins binding to the immunosuppressive drug FK506) and, more particularly, to parvulin (gene ppiC). Recent studies have shown that E. coli PPIase functions independently as an isomerase and as a chaperonin (3, 24).

Data dealing with antibodies to five identified antigens of H. pylori are interpreted in terms of the potential value these antigens may have as key compounds of an immunoblot assay that would allow differentiation of GDU disease from NUD. Differentiation of GDU from NUD seems to depend on factors such as age and population. Some reports provide evidence for the usefulness of the immunoblotting diagnosis of GDU in Brazilian children (25). Other authors claim that there is no association of specific H. pylori antigens with antibodies in adult patients suffering from various gastroduodenal pathologies in Germany (14) and Chile (20). Apparently, these contradictory findings reflect both the phenotypic heterogeneity of H. pylori and the specificity of the host immune response within various populations. However, a large-scale comparative immunoblotting study with sera from patients living in countries as geographically distant as the United States, China, and Peru has shown that, despite their diverse origins, all H. pylori strains show several major conserved bands in the range of 25 to 84 kDa (11). Other studies carried out in India and France also give evidence for relatively restricted, although differing, ranges of immunoreactive bands (2, 4). Therefore, it is possible that a pattern of immunoreactive bands may characterize, at least to some extent, the immune response to H. pylori within a given population, and this may be true irrespective of the H. pylori strain. In this latter regard, some 350 genes appear to be specific for H. pylori, but only 6 to 7% of them are considered to be strain specific (1, 27). This implies that some of the remaining 93 to 94% of these genes encode specific H. pylori antigens and/or epitopes that are common to all strains of this microorganism. The fact that we found similar antigen profiles in two H. pylori strains that were recognized by serum antibodies in patients from different regions of France supports this concept, although a prospective study with a greater number of H. pylori strains is needed to confirm our assumption.

The results presented in Tables 2 and 3 provide additional support that proteins such as catalase, groEL, and flagellin are strongly immunogenic but give nonspecific cross-reactions with antibodies to antigens from other gram-negative bacteria and, more particularly, Campylobacter jejuni (10, 12, 17, 21, 26). Catalase, groEL, and flagellin will therefore be of poor utility for the envisaged predictive test. Metabolic enzymes, such as identified β-ketoacyl-ACP S and PPIase, are considered to be promising candidates for a serological test kit because they have few cross-reactions with NUD sera and are more conserved than virulence factors such as CagA and VacA (14). Reported antibody frequencies to VacA, β-ketoacyl-ACP S, and PPIase make it possible to discriminate between the groups of NUD patients and ulcer patients, but it is rather unlikely that a single antigen could be sufficient as a highly reliable predictive tool. Neither the frequencies of positive sera for CagA and/or VacA nor the respective performance indices are sufficient as unique predictive markers for ulcer disease. This is due to the well-known fact that there exist H. pylori strains which are phenotypically negative for one or both antigens (5). In this regard, patterns including antibodies to β-ketoacyl-ACP S and PPIase could be promising for the prediction of GDU because their performance indices are close to 80%.

In conclusion, we identified the primary structure of five H. pylori protein antigens, and two of them—β-ketoacyl-ACP S and PPIase—are proposed for the first time as antigens of diagnostic interest. Antibody patterns detected by using these antigens may be useful in developing a diagnostic test designed to predict the clinical evolution of the H. pylori infection toward an ulcer disease within the adult population of France.

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