Evaluation of the Clinical Significance of homB, a Novel Candidate Marker of Helicobacter pylori Strains Associated with Peptic Ulcer Disease

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Background. homB codes for a putative Helicobacter pylori outer membrane protein and has previously been associated with peptic ulcer disease (PUD) in children.

Methods. A total of 190 H. pylori strains isolated from children and adults were studied to evaluate the clinical importance of the homB gene. In vitro experiments were performed to identify HomB mechanisms of bacterial pathogenicity.

Results. Characterization of the isolates demonstrated that homB was significantly associated with PUD in 86 children (odds ratio [OR], 7.64 [95% confidence interval {CI}, 2.65–22.05]) and in 32 adults ≤40 years of age (OR, 11.25 [95% CI, 1.86 – 68.13]). homB was correlated with the presence of cagA, babA2, vacAs1, hopQI, and oipA “on” genotype (P < .001). The HomB protein was found to be expressed in the H. pylori outer membrane and was noted to be antigenic in humans. H. pylori homB knockout mutant strains presented reduced ability to induce interleukin-8 secretion from human gastric epithelial cells, as well as reduced capacity to bind to these cells. Both of these functions correlated with the number of homB copies present in a strain.

Conclusion. homB can be considered a comarker of H. pylori strains associated with PUD. Moreover, results strongly suggest that HomB is involved in the inflammatory response and in H. pylori adherence, constituting a novel putative virulence factor.

Helicobacter pylori is the major causative agent of peptic ulcer disease (PUD), chronic active gastritis, gastric carcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [1]. Progression to more-severe disease occurs only in some infected individuals and depends on a number of factors, including host genetic susceptibility, environmental factors, and differences in the virulence of H. pylori strains. To date, the best recognized bacterial marker of H. pylori pathogenicity is cagA. The cagA gene is a marker for the cag pathogenicity island (cagPAI), which, when intact, encodes the components of a type IV secretion system responsible for delivering the CagA protein into the cytoplasm of the host cell, where it becomes tyrosine phosphorylated and initiates changes in cell signalling, coupled with induction of the proinflammatory interleukin (IL)–8 [2].

The onset of H. pylori infection occurs primarily during childhood [3], but severe gastroduodenal diseases appear primarily in adulthood, after a long-term infection [4, 5]. In young children, the development of a peptic ulcer occurs soon after H. pylori infection, suggesting that the implicated strain may be potentially more pathogenic. A putative ulcer-associated outer membrane protein (OMP), jhp0870, was recently identified, by means of subtractive hybridization, in an H. pylori strain isolated from a young child presenting with a duodenal ulcer [6].

The jhp0870 open-reading frame (ORF), homB, is 90% identical to another member of the H. pylori OMPs,
the jhp0649 ORF homA, with the differences between the two confined to the middle region of these ORFs. According to the sequenced H. pylori strains [7–9], both genes occupy well-conserved loci, with the homA locus occupied either by homA or homB, and with the homB locus either occupied by one of these ORFs or remaining empty with a segment displaying 96.8% similarity with homB. This finding suggests that homB can exchange positions with homA and, also, that the presence of homB may have resulted from gene duplication [10].

The clinical importance of homB was evaluated by analyzing its distribution and diversity in a collection of 190 H. pylori strains isolated from patients presenting only with PUD or gastritis, as well as its association with the previously described H. pylori virulence-associated genes. The exposed components, usually OMPs, can contribute to the colonization and persistence of H. pylori, by allowing the bacteria to adhere to gastric cells [11, 12]. The OMPs can also influence the pathogenesis of the disease process, by stimulation of the inflammatory response of the host [13]. Considering that the product of homB, HomB, is a virulence-associated OMP candidate, we also investigated its surface membrane localization and the immunological response of the patient. The contribution of HomB to the inflammatory response of H. pylori and to the bacterial adherence properties was also investigated in vitro.

**MATERIAL AND METHODS**

**Bacterial strains.** A total of 190 H. pylori clinical strains were cultured from antral biopsy specimens [14] (table 1). Two H. pylori sequenced strains, 26695 (ATCC 700392), positive for homA and negative for homB, and J99 (ATCC 700824), positive for both homA and homB, were used as reference strains [7, 8]. Genomic DNA was extracted as described elsewhere [6].

**Polymerase chain reaction (PCR)–based genotyping of virulence factors.** Genotyping of the virulence factors was performed by PCR for cagA [15], cagE, cagG, cagM [16], and the “cag empty site” [17], vacAs1 [18], babA2 [19], iceA [20], hopQ [21], homB, and homA [6]. The frame status of oipA, sabA, hopZ, and dupA was determined by sequencing [16, 22–24]. The sequence diversity of homB and homA was evaluated by sequencing entire genes [6].

**Construction of homB knockout mutant strains.** The pILL570/Hp 710ΔTnKm suicide plasmid containing a homB homologue disrupted by a kanamycin resistance gene cassette (supplied by I. Boneca, Microbiology Department of Institut Pasteur, Paris) was used for natural transformation of H. pylori strains. The correct and unique insertion of the kanamycin cassette in the target region was confirmed by PCR, sequencing, and Southern blot analysis. For further discussion of this procedure, see the Appendix (which is available only in the online version of the Journal).

**Two-dimensional gel electrophoresis (2DE) and analysis of protein spots.** A total of 200 µg of protein membrane pellets (i.e., sarcosine-insoluble outer membrane fractions) [25] of the H. pylori strain were separated by 2DE, by means of isolectric focusing performed with the use of an immobilized pH gradient strip with a nonlinear pH of 3–10 (GE Healthcare), followed by SDS-PAGE performed in 10% w/v polyacrylamide gels with the use of SE 600 Ruby equipment (GE Healthcare). Gels were either stained with Coomassie blue [26] or used for Western blot analysis.

Selected protein spots were excised from the gel and digested with trypsin. Peptides were analyzed by means of capillary liquid chromatography tandem mass spectrometry (LC-MS/MS). For further discussion of this procedure, see the Appendix (which is available only in the online version of the Journal).

**Preparation of recombinant HomB and immunoproteomics.** A recombinant HomB protein (rHpHomB) with the homB ORF of the J99 strain cloned (jhp0870, GenBank accession no. NC_000921) was prepared using a glutathione S-transferase (GST) gene fusion vector pGEX-4T-3 (GE Healthcare). The purified protein was probed with an anti-GST antibody (1:1000) (Chemicon Australia), to confirm its expression.

The 2DE or SDS-PAGE (purified rHpHomB) gels were transferred onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes and incubated with a pool of 10 serum samples obtained from patients (64.7% of whom were male; mean age ± SD, 51.2 ± 6.8 years) who had IgG antibodies against H. pylori. A pool of 5 serum samples obtained from H. pylori–uninfected individuals (53.3% of whom were male; mean age ± SD, 42.7 ± 5.4 years) who had an anti–H. pylori IgG antibody level below the threshold denoting positivity was used as negative control. The level of IgG antibodies against H. pylori was quantified using the serological test Pyloriset EIA-G III (Orion Diagnostica). For further discussion of this procedure, see the Appendix (which is available only in the online version of the Journal).
The level of significance was set at 5%, with the null hypothesis being rejected when \( P < .05 \).

### RESULTS

#### Prevalence of homB, homA, and other virulence genes in relation to clinical outcome

A total of 190 \textit{H. pylori} clinical strains (table 1) isolated from Portuguese children \((n = 84)\) and adults \((n = 106)\) were examined for the presence of the \textit{homB}, \textit{homA}, \textit{cagPAI}, \textit{vacAs1}, \textit{babA2}, \textit{iceA}, \textit{hopQ}, \textit{oipA}, \textit{sabA}, \textit{hopZ}, and \textit{dupA} genes.

Overall, with regard to the \textit{cagPAI} markers of these 190 patients, a concordance between the presence of the \textit{cagA}, \textit{cagE}, \textit{cagG}, and \textit{cagM} genes and the simultaneous absence of a \textit{cag} empty site product—or vice versa (i.e., absence of the genes and presence of the \textit{cag} empty site)—was observed in 135 \((71.0\%)\) of the 190 strains, suggesting that mixed infections with or without \textit{cagPAI} were not frequently observed in this group of strains. For 28 \((50.9\%)\) of the remaining 55 strains, a discrepancy was observed between the presence of \textit{cagA}, \textit{cagE}, \textit{cagG}, and \textit{cagM} genes, suggesting the presence of an incomplete island or mixed infections, of which only 5 were \textit{cagA} positive (17.9\%). According to these results, the \textit{cagA} gene was considered to be representative of an intact \textit{cagPAI} in this study. The prevalence and the univariate statistical significance of the association between the \textit{H. pylori} virulence genotypes, the \textit{homB} and \textit{homA} genotypes, and the clinical outcome are presented in table 2.

In strains isolated from children (31 with PUD and 53 with NUD), 5 genotypes were associated with PUD: \textit{cagA} \((P < .001; \text{OR}, 0.298)\) and \textit{vacAs1} \((P < .001; \text{OR}, 14.13)\), \textit{oipA} “on” genotype \((P < .001; \text{OR}, 14.06)\), \textit{hopQ}I \((P = .004; \text{OR}, 5.67)\), and \textit{homB} \((P < .001; \text{OR}, 7.64)\). Two genotypes were strongly associated with NUD: the \textit{sabA} “on” genotype \((P = .028; \text{OR}, 0.298)\) and \textit{homA} \((P = .006; \text{OR}, 0.263)\). Concerning \textit{dupA}, a PCR fragment corresponding to \textit{jhp0917–jhp0918} was detected in all tested strains, and the presence of an entire gene was confirmed by sequencing.

In strains isolated from adults (50 with PUD and 56 with NUD), only \textit{cagA} was significantly associated with PUD \((P = .05; \text{OR}, 2.28)\), indicating that \textit{cagPAI} is the major independent predictor of disease in the study population. Similarly to strains isolated in children, all isolates recovered from adults had an entire \textit{dupA} gene.

It is likely that environmental factors play a greater role in the development of PUD in older adult populations than in younger populations, as has been demonstrated for smoking and nonsteroidal inflammatory drug (NSAID) consumption [28]. Accordingly, the adult population was stratified by age, and 2 groups were formed: adults \(\leq 40\) years of age and those \(> 40\) years of age. In the group of patients \(\leq 40\) years of age \((n = 32)\), 17 of whom had PUD \((64.7\%\) of whom were male; mean age \(\pm SD, 33.5 \pm 6.5\) years) and 15 of whom had NUD \((58.3\%\) of whom...

<table>
<thead>
<tr>
<th>Genotype</th>
<th>\textit{PUD} associated ((n = 31))</th>
<th>\textit{NUD} associated ((n = 53))</th>
<th>(P^a)</th>
<th>OR (95% CI)</th>
<th>\textit{PUD} associated ((n = 50))</th>
<th>\textit{NUD} associated ((n = 56))</th>
<th>(P^a)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{cagA}^+</td>
<td>23(74.2)</td>
<td>9(17.0)</td>
<td>.001</td>
<td>14.06 (4.78–41.29)</td>
<td>34(68.0)</td>
<td>27(48.2)</td>
<td>.050</td>
<td>2.28 (1.03–5.04)</td>
</tr>
<tr>
<td>\textit{vacAs1}</td>
<td>23(74.2)</td>
<td>10(18.9)</td>
<td>.001</td>
<td>14.13 (4.75–42.04)</td>
<td>31(62.0)</td>
<td>26(46.4)</td>
<td>.116</td>
<td>1.99 (0.91–4.35)</td>
</tr>
<tr>
<td>\textit{oipA “on”}</td>
<td>26(83.9)</td>
<td>15(28.0)</td>
<td>.004</td>
<td>5.67 (1.73–18.53)</td>
<td>30(60.0)</td>
<td>28(50.0)</td>
<td>.067</td>
<td>2.13 (0.96–4.69)</td>
</tr>
<tr>
<td>\textit{hopQ}I</td>
<td>17(54.8)</td>
<td>15(28.0)</td>
<td>.004</td>
<td>5.67 (1.73–18.53)</td>
<td>29(58.0)</td>
<td>25(44.6)</td>
<td>.163</td>
<td>2.09 (0.82–5.35)</td>
</tr>
<tr>
<td>\textit{babA2}^+</td>
<td>9(29.0)</td>
<td>6(11.3)</td>
<td>.074</td>
<td>3.21 (1.01–10.13)</td>
<td>25(50.0)</td>
<td>18(33.3)</td>
<td>.076</td>
<td>2.11 (0.96–4.64)</td>
</tr>
<tr>
<td>\textit{iceA}1</td>
<td>16(51.6)</td>
<td>14(26.4)</td>
<td>.562</td>
<td>0.603 (0.18–2.00)</td>
<td>9(18.0)</td>
<td>10(17.9)</td>
<td>1.000</td>
<td>1.13 (0.40–3.18)</td>
</tr>
<tr>
<td>\textit{hopZ “on”}</td>
<td>30(60.0)</td>
<td>27(48.2)</td>
<td>.328</td>
<td>1.56 (0.72–3.37)</td>
<td>30(60.0)</td>
<td>27(48.2)</td>
<td>.328</td>
<td>1.56 (0.72–3.37)</td>
</tr>
<tr>
<td>\textit{sabA “on”}</td>
<td>7(22.6)</td>
<td>30(56.6)</td>
<td>.028</td>
<td>0.298 (0.11–0.83)</td>
<td>28(56.0)</td>
<td>39(69.6)</td>
<td>.56 (0.25–1.23)</td>
<td>39(69.6)</td>
</tr>
<tr>
<td>\textit{homB}^+</td>
<td>25(80.6)</td>
<td>18(33.3)</td>
<td>.006</td>
<td>0.263 (0.10–0.67)</td>
<td>25(50.0)</td>
<td>18(33.3)</td>
<td>.006</td>
<td>0.263 (0.10–0.67)</td>
</tr>
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</table>

\( ^a \) \text{P values were determined using Fisher’s exact test.}
were male; mean age $\pm$ SD, 34.7 $\pm$ 5.4 years), homB was associated with PUD (88.2% vs. 40.0% [P = .008]; OR, 11.25 [95% CI, 1.86–68.13]). Compared with strains isolated from patients with NUD, strains isolated from patients with PUD had a higher prevalence of cagA (82.4% vs. 53.3%), vacAs1 (82.4% vs. 53.3%), oipA “on” genotype (88.2% vs. 60.0%), and hopZ “on” genotype (70.6% vs. 40.0%), although the differences were not statistically significant. homA was a marker for NUD (60.0% vs. 17.6% [P = .027]; OR, 7.0 [95% CI, 1.11–50.44]). In the group of adults $>$ 40 years of age, no genotype was significantly associated with clinical outcome (not shown).

**Association of homB and homA with other virulence genes.** Globally, the presence of homB was associated with cagA ($P < .001$), vacAs1 ($P < .001$), babA2 ($P < .001$), and hopQII ($P < .001$), and oipA “on” genotype ($P < .001$), whereas homA was strongly associated with cagA” ($P = .01$), vacAs2 ($P < .001$), babA2” ($P = .001$), hopQII ($P < .001$), and oipA “off” ($P < .001$). The presence of homB was also significantly associated with the absence of homA ($P < .001$). Figure 1 illustrates the association of homB with cagA and vacAs1 (figure 1A), as well as that of homA with cagA and vacAs2 (figure 1B). The same degree of association was found when strains isolated from children and adults were analyzed separately and, also, when strains of different clinical origins were considered (data not shown).

**Binary logistic regression analysis.** A binary logistic regression analysis was performed to evaluate which combinations of genotypes presented the most discriminatory capacity to distinguish between PUD and NUD (table 3). In H. pylori strains isolated from children, combinations of genotypes including homB and the other genotypes presenting the highest risk for PUD were tested. The analysis of the adjusted OR showed that, when present in a model, the cagA gene was the most important predictor of PUD. Both vacAs1 and oipA “on” genotype when combined with homB also presented the highest association.

**Table 3. Binary logistic regression analysis to discriminate between peptic ulcer disease and nonulcer dyspepsia, using as predictors different associations of the Helicobacter pylori virulence genotypes, in strains isolated from Portuguese children and from adults aged $<40$ years.**

<table>
<thead>
<tr>
<th>Patient group, model</th>
<th>$P$</th>
<th>Adjusted OR (95% CI)</th>
<th>Area under the ROC curve % ($P$)</th>
<th>H-L test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Children (n = 84)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA*</td>
<td>.019</td>
<td>5.94 (1.34–26.23)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vacAs1</td>
<td>.062</td>
<td>4.12 (0.93–18.22)</td>
<td>83.4 ($&lt; .001$) 0.056</td>
<td></td>
</tr>
<tr>
<td>cagA*</td>
<td>.001</td>
<td>8.26 (2.58–26.44)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vacAs1</td>
<td>.054</td>
<td>3.31 (0.98–11.18)</td>
<td>83.4 ($&lt; .001$) 0.005</td>
<td></td>
</tr>
<tr>
<td>hopQII</td>
<td>.001</td>
<td>8.42 (2.41–29.47)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hopZ +</td>
<td>.182</td>
<td>2.43 (0.66–8.98)</td>
<td>81.6 ($&lt; .001$) 0.438</td>
<td></td>
</tr>
<tr>
<td>oipA “on”</td>
<td>.003</td>
<td>6.12 (1.83–20.40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>homB*</td>
<td>.026</td>
<td>3.81 (1.17–12.36)</td>
<td>82.1 ($&lt; .001$) 0.209</td>
<td></td>
</tr>
<tr>
<td><strong>Adults (n = 32)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cagA*</td>
<td>.366</td>
<td>2.33 (0.37–14.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hopB*</td>
<td>.020</td>
<td>9.00 (1.41–57.44)</td>
<td>77.8 (.007) 0.885</td>
<td></td>
</tr>
<tr>
<td>vacAs1</td>
<td>.366</td>
<td>2.33 (0.37–14.65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hopB*</td>
<td>.020</td>
<td>9.00 (1.41–57.44)</td>
<td>77.8 (.007) 0.885</td>
<td></td>
</tr>
<tr>
<td>oipA “on”</td>
<td>.366</td>
<td>3.00 (0.40–22.77)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hopZ “on”</td>
<td>.066</td>
<td>2.06 (0.89–36.17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>homB*</td>
<td>.008</td>
<td>15.81 (2.06–121.61)</td>
<td>82.0 (.002) 0.762</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; H-L, Hosmer-Lemeshow; OR, odds ratio; ROC, receiver operating characteristic.

* Reference category is the absence of the H. pylori virulence genotypes.

b As determined by the Wald test.
and most significant adjusted OR when combined with homB. Analysis of the area under the receiver operating characteristic curves showed that all models proposed presented a high and similar discriminatory capacity to distinguish between PUD and gastritis, whereas the goodness of fit of each model to the data, as evaluated by the Hosmer-Lemeshow test, showed that only the models vacA*1 with homB and oipA “on” with homB showed a good adjustment (H-L test, >0.05).

In strains isolated from adults ≤40 years of age, homB was the most important predictor of PUD, showing the highest value for the adjusted OR in every model considered and, therefore, corroborating the univariate analysis. Overall, the best model for discriminating between PUD and NUD in young adults was the combination of homB with hopZ “on genotype.”

**Diversity in homB and homA genes.** The presence of homB and homA and their genomic positions were analyzed in 190 *H. pylori* clinical strains. At least 1 of these 2 genes was always present in the genome of the clinical strains, with different combinations observed: a single-copy genotype (homA or homB) or a 2-copy genotype (either 2 copies of homA or homB, or 1 copy of each gene). The entire nucleotide sequence of both the homB and homA genes was determined in 58 clinical strains, which were randomly chosen, presenting different homB/homA combinations: 13 strains had 2 copies of homA, 6 had 2 copies of homB, 16 had 1 copy of homB, 10 had 1 copy of homA, and 13 had 1 copy of each gene. Both genes presented a variable length: from 1974 to 2025 bp for homB and from 1941 to 1998 bp for homA. In cases where only 1 copy of homB or homA was present, the gene was present in the jhp0649 locus, whereas a conserved intergenic space was observed at the jhp0870 locus, according to the numbering of the J99 strain [10]. When 2 copies were found in the same strain, genes were present indifferently in one of these loci. Whenever duplicates of the same gene were present in a single strain, their identity varied between 98% and 99%.

The presence of homB and/or homA in a single- or 2-copy genotype varied significantly among PUD- and NUD-associated strains isolated from children and young adults. The single-copy genotype (homB or homA) was more frequently associated with NUD (79.4% vs. 37.5%), whereas the 2-copy genotype (homB/homA, homB/homB, or homA/homA) was more frequently associated with PUD (62.5% vs. 20.6%). More specifically, a single-copy of homA was the genotype most frequently associated with NUD (61.8% vs. 10.4% [P < .001]; OR, 13.89 [95% CI, 4.49–45.99]), whereas 2 copies of homB was the genotype most strongly associated with PUD (39.9% vs. 11.8% [P = .001]; OR, 4.91 [95% CI, 1.77–14.02]).

**Evaluation of HomB surface expression and antigenicity.** Nine spots were identified as being expressed differentially among the 2DE OMP fractions of the wild-type (wt) strain 1776/05 and the corresponding homB mutant (figure 2A). In 2 spots, the *H. pylori* putative OMPs HomD and HopN, which have not yet been described in *H. pylori* proteomic studies, were identified by LC-MS/MS analysis. The other 6 spots corresponded to previously described surface proteins, validating the preparation of the OMP extracts [30–33] (table 4) (for further discussion, see the Appendix, which is available only in the online version of the *Journal*). The remaining spot, which had an isoelectric point and a molecular weight similar to those deduced for HomB, was identified in the 2DE of the wt strain, and the LC-MS/MS analysis confirmed that this spot was the HomB protein. This spot was absent in the homB knockout mutant on 2DE (not shown).

The immunoblot showed that HomB was immunoreactive to serum samples obtained from *H. pylori*-positive patients (figure 2B). This result was confirmed by the immunoreaction with the rHPHomB (figure 2C). Neither the 2DE HomB spot nor the rHPHomB showed reactivity with the pool of serum samples obtained from *H. pylori*-negative patients (data not shown). The Western blot of the 2DE also showed that HomD and HopN were immunoreactive (figure 2B).

**In vitro cellular IL-8 secretion, according to the homB status of the strains.** The level of IL-8 secretion was evaluated for the 2 *H. pylori* reference strains (26695 and J99) and the 7 pairs of wt and corresponding homB mutants (figure 3A). IL-8 secretion by epithelial cells is dependent on the presence of a functional cagPAI [35, 36]. As expected, the mean amount of IL-8 secretion was significantly higher in cagPAI-positive strains (mean ± SD, 772.1 ± 215.7 pg/mL) than in cagPAI-negative strains (mean ± SD, 102.5 ± 12 pg/mL). Concerning the 5 cagPAI-positive strains, IL-8 levels obtained with the homB single mutants were significantly lower than those obtained with the parent strains (figure 3A). For strain 417/02, which carried 2 copies of homB, the IL-8 level obtained for the double mutant was significantly lower than that observed for the corresponding single mutant. A significant reduction in IL-8 secretion was also observed for the strain 565/99, 1 of the 2 cagPAI-negative homB single mutants.

**Adherence according to the homB status of the strains.** All of the *H. pylori* strains tested were able to adhere to the human cells (figure 3B). The homB disruption of strains 771/99 and 36/00 did not lead to a significant modification in adherence properties. Concerning strains 1776/05, 559/02, 351/99, and 565/99, the inactivation of the single genomic copy of homB led to a significant decrease in adherence. For strain 417/02, which carried 2 copies of homB, the disruption of 1 copy of homB led to a significant decrease in adherence, compared with the wt strain, and this decrease was more important when the 2 copies were disrupted, compared with the single mutant strain (figure 3B).

**DISCUSSION**

The present study attempts to bring insight to the clinical significance of a novel *H. pylori* ulcer marker candidate, the homB gene, coding for an OMP, aiming to clarify its role as a virulence...
factor associated with the development of PUD. The distribution of \textit{homB} in clinical \textit{H. pylori} strains showed that \textit{homB} was significantly associated with PUD in children and young adults (≤40 years of age). In contrast, \textit{homA}, the gene to which \textit{homB} was 90% similar, was a marker for NUD in those strains. In addition, \textit{cagA}, \textit{vacAs1}, \textit{hopQI}, and \textit{oipA} “on” genotypes were also strongly associated with PUD in children, whereas, in adults, only \textit{cagA} was a marker for PUD. Several studies reported such differences in strains infecting the mucosa of adults and children [6, 37–40]. On the other hand, the fact that \textit{homB} was a marker for PUD in children and young adults suggests that disease severity in younger populations is more closely related to the virulence of the infecting strain than to environmental factors, such as smoking and high NSAID consumption, which play a major role in older populations [28]. Nevertheless, these results were obtained with small groups of strains, particularly those isolated from young adults, and they therefore require future validation. Overall, it should be emphasized that, among the \textit{H. pylori} virulence genotypes analyzed, \textit{cagA}, as a marker for the \textit{cag} PAI, was the only predictor of PUD in both children and adults; this finding is in agreement with other studies and thereby reconfirms that \textit{cagPAI} is one of the major \textit{H. pylori} virulence factors [41, 42].

The overall profile of the association of \textit{homB} with clinical outcome is supported by the correlation of this factor with the other \textit{H. pylori}–associated virulence genotypes, independent of...
homB is observed much more frequently in highly virulent strains, suggesting that homB status may be useful in determining a genetic pattern associated with a higher degree of H. pylori pathogenicity.

The homA gene, a 90% homologue and duplicate of homB, was strongly associated with both NUD and the less virulent genotypes. Moreover, 2 copies of homB in a single strain was the genotype most strongly associated with ulcerogenic strains, whereas the strongest marker of nonulcerogenic strains was 1 copy of homA. These results suggest that, although homB and homA share a high similarity, they probably evolved for different functions, with homB most likely contributing more to the proinflammatory properties of H. pylori strains. Other H. pylori genes encoding OMPs are present in duplicate copies within the same strain, and functional diversification has been observed; for example, babA codes for the blood group antigen-binding adhesin BabA, whereas the babB product is associated with a nonbinding phenotype [11].

The existence of duplicated genes in a single genome can lead to gene conversion, by recombination between loci, leading to the duplication of a unique gene [11, 43, 44]. Sequence analysis of homB and homA, which showed that they are present as single- or 2-copy genotypes in conserved loci, suggests that the duplication phenomenon can occur between these 2 genes. Thus, it seems that different strains may duplicate different genes, and whether this constitutes a random event or confers any biological advantage to the bacterial cells remains unknown.

The age of the patient and the clinical origin of the strain. Thus, homB is observed much more frequently in highly virulent strains, suggesting that homB status may be useful in determining a genetic pattern associated with a higher degree of H. pylori pathogenicity.

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The observation that the 2-copy genotype was more prevalent in strains associated with PUD than in strains associated with NUD points to a nonrandom event of duplication, suggesting a biological advantage, either by facilitating elevated expression of a particular protein or by facilitating adaptation to an individual host.

HomB was shown to be expressed at the H. pylori outer membrane and recognized by human serum samples, indicating that it possesses surface-exposed regions. The inactivation of homB caused a significant reduction in IL-8 production, a phenomenon observed in all of the cagPAI-positive strains and in 1 of the 2 cagPAI-negative strains tested. The double knockout of homB had a stronger reduction effect, suggesting that both HomB proteins are active and share the same function. This is in accordance with the finding that the presence of 2 copies of homB in a single strain was the genotype most frequently found in ulcerogenic strains. It should, however, be emphasized that the level of reduction in IL-8 secretion obtained with homB mutants was low compared with what was previously observed with cagPAI mutants [35, 36]. These results suggest that, in the presence of cagPAI, HomB is able to promote an in vitro proinflammatory response by gastric epithelial cells. This hypothesis is supported by the finding that homB is strongly associated with cagA, as well as with other H. pylori virulence factors, because it has been described for other H. pylori OMPs [19, 45], and it suggests a shared selective pressure favoring the expression of multiple virulence determinants.

Helicobacter pylori–related IL-8 induction requires attachment of live bacteria to epithelial cells [46, 47], a prerequisite required for CagA translocation and consequent induction of IL-8 secretion [48]. Accordingly, it is likely that the mechanism underlying the involvement of HomB in inflammation is bacterial adherence. Indeed, it was demonstrated that H. pylori wt strains adhered to human gastric epithelial cells, whereas the corresponding homB knockout mutant strains showed significantly reduced binding. However, the fact that the disruption of homB did not completely abolish adherence suggests that HomB is not the major OMP involved in this mechanism. Indeed, adherence of H. pylori to the human gastric epithelium is mediated by the BabA major adhesin to the fucosylated Lewis b blood group antigen [11]. Because the AGS cells express variable levels of Lewis b blood group antigen [49], it would be interesting to test other cell lines—for example, the gastric epithelial Kato III cells deficient in Lewis b epitope—for which H. pylori adherence is independent of the BabA–Lewis b binding [49].

In conclusion, homB can be considered a comarker of H. pylori strains associated with PUD. HomB is immunogenic and is likely involved in the inflammatory response and in adherence, suggesting its contribution to the development of a more severe clinical outcome. Overall, results suggest that homB is a novel H. pylori putative virulence factor.

Acknowledgments

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SUPPLEMENTARY INFORMATION REGARDING MATERIALS AND METHODS

Construction of homB knockout mutant strains. The homB knockout mutant strains were constructed by insertion of a kanamycin cassette by double homologous recombination. A suicide plasmid containing the targeted gene (HP0710, a homB homologue) disrupted by a kanamycin resistance gene cassette (pILL570Hp710ΔTnKm plasmid) was used for natural transformation of Helicobacter pylori. Transformation was performed on an agar surface. In brief, 24 h after inoculation, bacteria were harvested from an agar plate, suspended in 200 μL of Brucella broth, spread on an agar base supplemented with 10% horse blood, and incubated at 37°C for 5 h. Approximately 50 ng of plasmid DNA was spotted directly onto the inoculated agar, and incubation was continued for 10 h. The incubated mixture then was spread on a nonselective medium for 18 h (Columbia agar supplemented with 10% horse blood). Bacteria then were suspended in 300 μL of Brucella broth, spread on selective plates of Columbia agar supplemented with 10% horse blood and 20 μg/mL kanamycin, and incubated at 37°C in a microaerobic atmosphere for 5 days.

For construction of the double mutant, a second transformation experiment was performed on a single mutant, with the homB gene localized at the previously inactivated jhp0649 locus. Double-mutant colonies were selected on Columbia agar supplemented with 10% horse blood and 250 μg/mL kanamycin.

The correct insertion of the kanamycin cassette in the target region was confirmed by polymerase chain reaction (PCR) performed with external primers located on the flanking genes and, subsequently, by sequencing. Southern blot hybridization was also performed to verify that there was no illegitimate insertion of the kanamycin cassette elsewhere in the H. pylori genome. A 172-bp probe was prepared from the pILL570Hp710ΔTnKm plasmid with the primers F1-clone (ACTAATGCTTGAAACCCAG)/R1-clone (CTCCAAATTCACTGTTCCTTG) and was used to hybridize to the genomic DNA digested with HindIII (Promega). The signal was detected using the DIG Luminescent Detection Kit (Roche Diagnostics), according to the manufacturer’s instructions. A single fragment of 1700 bp was detected for both the single and double mutants (data not shown). In addition, PCR performed with the internal primers F1/-R1-jhp0870/jhp0649 confirmed that neither homB nor homA was present in the transformed strain (data not shown).

Membrane protein preparation. Sarcosine-insoluble outer membrane fractions, from the wild-type 1776/05 strain and the corresponding homB knockout mutant strain, were prepared as described elsewhere [25]. Protein quantification was done according to the Bradford method, by use of the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories).

Two-dimensional gel electrophoresis (2DE). A total of 200 μg of protein membrane pellets of each H. pylori strain was precipitated with 20% trichloroacetic acid, washed in ice-cold acetone, and solubilized in isoelectric focusing (IEF) buffer—7 mol/L urea, 2 mol/L thiourea, 4% w/v 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate, 60 mmol/L 1,4-dithiоerythriol, 0.75% w/v ampholines (pH 3.5–10.0), 0.25% w/v ampholines pH 4–6—at 37°C for 30 min. The samples were clarified by centrifugation performed at 12,000 g for 5 min, and the supernatants containing the membrane protein extracts were loaded onto 7-cm immobilized pH gradient (IPG) strips (Immobiline DryStrips) with a nonlinear pH of 3–10 (GE Healthcare). IEF was performed via stepwise voltage increments from 100 V to 6500 V, to a total of 29,000 Vh in Protein IEF Cell equipment (Bio-Rad Laboratories), after an active rehydration at 50 V for 12 h. For the second dimension, IPG strips were equilibrated in equilibration solution (50 mmol/L Tris-HCl [pH 8.8], 6 mol/L urea, 30% w/v glycerol, 2% w/v SDS, and traces of bromophenol blue) with 2% w/v 1,4-dithiоerythriol for 15 min and with 2.8% w/v iodoacetamide for another 15 min. The IPG strips were sealed with agarose (0.5% in running buffer) in 10% w/v polyacrylamide gels (1-mm thick) and were run at 50 V for 16 h, by use of SE 600 Ruby equipment (GE Healthcare).

Tryptic digestion of 2DE gel spots and mass spectrometric analysis. After gel analysis, selected protein spots were excised from the gel for mass spectrometry analysis and were kept in microtubes at −20°C until they were analyzed. The protein spots were cut with a razor blade into small cubes and were washed 5 times with 40% w/v 1-propanol, followed by 5 washes with 50% w/v acetonitrile and 0.1 mol/L NH4HCO3. The gel pieces were shrunk with 50 μL of 100% w/v acetonitrile and were dried completely in a SpeedVac. The dried gel pieces were rehydrated with 10 μL of 50 mmol/L NH4HCO3 containing 125 ng of trypsin (Promega). Once the gel pieces were impregnated with the trypsin solution, an additional 50 mmol/L NH4HCO3 was added until the gel pieces were completely immersed in liquid. Digestion was performed at 37°C overnight. After digestion was completed, the supernatant was transferred into a new tube, and the gel pieces were extracted with 50 μL of 50% w/v acetonitrile and 0.1% w/v formic acid, and the extract was combined with the digest. The digest was dried in a SpeedVac and redissolved in 25 μL of 0.1% w/v formic acid containing 2% w/v acetonitrile. The peptides were analyzed by capillary liquid chromatography on a Magic C18 100-μm × 10-cm reverse-phase column (Thermo Fisher Scientific) that was connected to an LTQ-Orbitrap instrument (Thermo Fisher Scientific). A linear gradient from 5% to 75% w/v solvent B (80% w/v acetonitrile containing 0.1% w/v formic acid) was delivered with a Rheos 2200 high-performance liquid chromatography system (Thermo Fisher Scientific) at 50 mL/min. A precolumn flow...
splitter reduced the flow through the column to ~500 nL/min. The eluting peptides were ionized by electrospray ionization, and the peptides were automatically selected and fragmented in the ion trap.

**Immunoproteomics.** 2DE gels were transferred to nitrocellulose membranes by use of a TE 62 Transfer Unit (GE Healthcare) apparatus at 400 mA for 90 min. Membranes were blocked with 5% skim milk in 0.1% w/v PBS-Tween (PBS-T) at room temperature for 1 h, and they were incubated with patients’ serum samples (1:400 in PBS-T) at 4°C for 16 h. Incubation with the secondary antibody anti–human IgG horseradish peroxidase from sheep (GE Healthcare) (1:1500 in PBS-T) was performed at room temperature for 1 h, and development was done using the ECL Western-blot kit (GE Healthcare).

**Data analysis.** Individual tandem mass spectrometry spectra, recorded by Xcalibur software (version 2.0; ThermoFinnigan), were compared, by use of TurboSequest software (BioWorks version 3.1; Thermo Electron), with the protein data bank and a subset of the National Center for Biotechnology Information database consisting of protein sequences from *H. pylori*. The search parameters were as follows: minimum cross correlation coefficients of 1.5, 2.0, and 2.5 for singly-, doubly-, and triply-charged precursor ions, respectively, with \( \Delta C_n > 0.1 \).
Table 4. Proteins identified, by mass spectrometry, in the 9 spots differentially expressed in 2-dimensional gel electrophoresis of the outer membrane proteome of *2 Helicobacter pylori* 1776/05 strains: the *homB* knockout mutant and the wild type.

<table>
<thead>
<tr>
<th>Protein identified</th>
<th>Protein annotation</th>
<th>Gene name</th>
<th>Theoretical Mr (kDa)</th>
<th>Matching peptides, no.</th>
<th>Xcorr (charge)</th>
<th>Sequence coverage, %</th>
<th>Antigenic spot (status)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGT</td>
<td>γ-GGT</td>
<td>HP1118</td>
<td>61.151</td>
<td>4</td>
<td>4.49 (3+)</td>
<td>10.0</td>
<td>No(B)</td>
</tr>
<tr>
<td>AtpA</td>
<td>Predicted AtpA</td>
<td>HP1134</td>
<td>55.143</td>
<td>3</td>
<td>3.3 (2+)</td>
<td>7.4</td>
<td>Yes(A)</td>
</tr>
<tr>
<td>FlgE</td>
<td>Flagellar hook protein</td>
<td>HP0870</td>
<td>76.207</td>
<td>17</td>
<td>6.16 (2+)</td>
<td>32</td>
<td>Yes(B)</td>
</tr>
<tr>
<td>FlgA</td>
<td>Flagellin A</td>
<td>HP0601</td>
<td>53.153</td>
<td>5</td>
<td>5.33 (2+)</td>
<td>15</td>
<td>Yes(B)</td>
</tr>
<tr>
<td>PpiC</td>
<td>Predicted PpiC*</td>
<td>HP0175</td>
<td>34.031</td>
<td>3</td>
<td>2.98 (2+)</td>
<td>15.5</td>
<td>No(B)</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>Predicted CR JHP0216</td>
<td>HP0231</td>
<td>29.459</td>
<td>3</td>
<td>4.06 (2+)</td>
<td>11</td>
<td>Yes(B)</td>
</tr>
<tr>
<td>HomD</td>
<td>Predicted OMP HomD</td>
<td>HP1453</td>
<td>82.360</td>
<td>2</td>
<td>2.55 (2+)</td>
<td>3.1</td>
<td>Yes(B)</td>
</tr>
<tr>
<td>HomB</td>
<td>Predicted OMP HomB</td>
<td>...</td>
<td>74.192</td>
<td>2</td>
<td>2.77 (2+)</td>
<td>6</td>
<td>Yes(A)</td>
</tr>
<tr>
<td>HopN</td>
<td>Predicted OMP HopN</td>
<td>HP1342</td>
<td>75.740</td>
<td>2</td>
<td>2.76 (3+)</td>
<td>5</td>
<td>Yes(A)</td>
</tr>
</tbody>
</table>

**NOTE.** AtpA, adenosine triphosphate synthase F1 (subunit alpha); CR, coding region; GGT, glutamyltranspeptidase; γ-GGT, gamma-glutamyltranspeptidase; LC-MS/MS, liquid chromatography tandem mass spectrometry; M, molecular weight; OMP, outer membrane protein; PpiC, peptidyl-prolyl cis-trans isomerase C; Xcorr, cross-correlation score.

- Gene product/function and theoretical Mr determined according to Boneca [34].
- According to the numbering of strains 26695, J99, and HPAG1, respectively [7–9].
- Only peptides with an Xcorr >1.5 (single charge), 2 (double charge), or 2.5 (triple charge) were considered. In all cases, ΔCn > 0.1.
- Spots apparently absent (A) or less intense (B) in the 2-dimensional gel electrophoresis of the proteome of the *Helicobacter pylori homB* knockout mutant strain, compared with those of the wild-type strain.
- Involved in protein maturation.
- With no homologue in the databases.