Alanine-Threonine Polymorphism of Helicobacter pylori RpoB Is Correlated with Differential Induction of Interleukin-8 in MKN45 Cells

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Geographical differences in the genetic diversity of Helicobacter pylori isolates were examined by analyzing rpoB sequences. An extremely high level of allelic diversity among H. pylori strains was found. The rpoB sequences of Asian and non-Asian (North and South American, European, and South African) strains were found to differ. An amino acid polymorphism (alanine and threonine RpoB types) was found at the 497th residue by deduced amino acid analysis. RpoB with a threonine residue (RpoBThr) was uniquely present in East Asian countries, and two-thirds of the H. pylori isolate population in this region was RpoBThr; however, this type was rare or absent in Western countries, where RpoBAla predominated. RpoBThr strains induced a much larger amount of interleukin-8, a chemokine that plays an important role in chronic inflammation, than RpoBAla strains in cultured MKN45 cells.

Helicobacter pylori is disproportionately acquired during childhood and persists in its host for life. H. pylori infection typically leads to chronic inflammation of the gastric mucosa, and this is accompanied by mucosal damage, including the loss of acid-secreting parietal cells and the development of mucous cell metaplasia (30). H. pylori carriers have a higher risk of gastric diseases like gastric cancer, which is the second most common malignancy worldwide, and is particularly common in East Asian countries, such as Korea and Japan (8, 22). However, H. pylori factors involved in gastric carcinogenesis are not well understood.

The presence of the cytotoxin-associated gene A (cagA) of H. pylori has been proposed to be an important risk factor for the development of H. pylori-mediated gastric cancer (7). Recently, it was suggested that Src homology 2-containing tyrosine phosphatase (SHP-2) is an intracellular target of CagA protein (9) and that the prevalent CagA type in East Asian countries binds more strongly to SHP-2, and thus induces more cellular morphological changes, than the CagA type prevalent in Western countries (10). Moreover, it has been suggested that this difference may be correlated with the striking difference in the incidence of gastric cancer in these two geographical areas (10). However, even though nearly 100% of Korean and Japanese isolates possess cagA and express the East Asian type of CagA, relatively few infected individuals develop peptic ulcer or gastric cancer (6). The reason for this remains unresolved (28).

Phylogenetic analysis based on amino acid sequences often provides more significant information than analysis based on the nucleotide sequences of protein-coding genes (8, 20). However, such analyses have not been performed in previous population studies with cagA, oipA, or other housekeeping genes (5, 17, 29, 31). Thus, to test the hypothesis that certain H. pylori strains in Asia are uniquely prone to cause chronic inflammation and metaplastic changes in the gastric mucosa, we studied the population structure of H. pylori isolates from several countries by analyzing rpoB sequences. This allowed us to analyze the H. pylori population by both nucleotide and amino acid sequence analyses. rpoB encodes the β-subunit of RNA polymerase and is a highly conserved housekeeping gene. Comparisons of rpoB sequences have previously been used for phylogenetic analysis and for the differential identification of bacteria (14, 15, 19, 34).

MATERIALS AND METHODS

Bacterial strains. The DNAs of 535 clinical H. pylori isolates from 12 countries (Table 1) were analyzed; H. cinaedi was used as an outgroup. The strains, which were provided by D. Y. Graham, had been obtained from patients who had signed informed consent forms approved by institutional review boards in the United States.

Preparation of DNA. H. pylori DNAs were extracted from cultured bacteria and gastric biopsy specimens by the bead beater-phenol extraction method (14). A loopful of a culture of each isolate was suspended in 200 μl of TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl [pH 8.0]) and placed in a 2.0-ml
TABLE 1. Prevalence of \( H. \) pylori types on the basis of the \( rpoB \) amino acid, by country

<table>
<thead>
<tr>
<th>Geographical region and country</th>
<th>No. (%) of strains (( n = 535 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>East and Southeast Asia</td>
<td>RpoB(^{\text{Thr}}) type</td>
</tr>
<tr>
<td>Korea</td>
<td>129 (23.4)</td>
</tr>
<tr>
<td>Japan</td>
<td>119</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>7</td>
</tr>
<tr>
<td>Taiwan</td>
<td>1</td>
</tr>
<tr>
<td>Thailand</td>
<td>2</td>
</tr>
<tr>
<td>North America</td>
<td>77 (98.7)</td>
</tr>
<tr>
<td>United States</td>
<td>72 (1.3)</td>
</tr>
<tr>
<td>Canada</td>
<td>5</td>
</tr>
<tr>
<td>South America</td>
<td>44 (93.6)</td>
</tr>
<tr>
<td>Colombia</td>
<td>42 (3.6)</td>
</tr>
<tr>
<td>Brazil</td>
<td>2</td>
</tr>
<tr>
<td>Europe</td>
<td>8 (100)</td>
</tr>
<tr>
<td>France</td>
<td>4</td>
</tr>
<tr>
<td>Italy</td>
<td>4</td>
</tr>
<tr>
<td>South Africa</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>262 (273)</td>
</tr>
</tbody>
</table>

\(^a\) The individual was born in Vietnam and immigrated to the United States.

- **DNA amplification.** PCR was performed with forward primer HF (5'-A CTTAAGGATGAAGATAT-3') and reverse primer HR (5'-ATTTTGGGCACTTGTTGGGTTG-3') to amplify \( rpoB \) DNA (458 bp) containing the Rifr region (16). Template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (AccuPower PCR PreMix; Bioneer, Daejeon, South Korea) containing 1 U of Taq DNA polymerase, each deoxynucleoside triphosphate at a concentration of 250 \( \mu \)M Tris-HCI (pH 8.3), 40 mM KCl, 1.5 mM MgCl\(_2\), and gel loading dye. The volume was adjusted to 20 \( \mu \)l with distilled water. The reaction mixture was then subjected to 30 cycles of amplification (30 s at 94\(^\circ\)C, 45 s at 52\(^\circ\)C, and 45 s at 72\(^\circ\)C), followed by a 5-min extension at 72\(^\circ\)C (model 9600 thermocycler; Perkin-Elmer Cetus). The PCR products were electrophoresed on a 1.2% agarose gel and purified by using a QIAEX II gel extraction kit (Qiagen).

- **Nucleotide sequencing.** The nucleotide sequences (363 bp) of the purified PCR products were directly determined with forward and reverse primers, using an Applied Biosystems model 373A automatic sequencer and a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom). For the sequencing reaction, 60 ng of PCR-amplified DNA, 3.2 pmol of either the forward primer or the reverse primer, and 8 \( \mu \)l of BigDye Terminator RRmix (part no. 43015512114; Perkin-Elmer Applied Biosystems) were mixed and adjusted to a final volume of 20 \( \mu \)l with distilled water. The reaction was run with 5% (vol/vol) dimethyl sulfoxide for 30 cycles of 15 s at 95\(^\circ\)C, 10 s at 50\(^\circ\)C, and 4 min at 60\(^\circ\)C. Both strands were sequenced as a cross-check. PCR and nucleotide sequencing of \( cagA \) were also performed as described previously (10, 32).

**Sequence alignment and phylogenetic tree.** The partial \( rpoB \) sequences (363 bp) were aligned by multiple-alignment program in the MegAlign program (Windows version 3.12e; DNASTAR, Madison, Wis.) and the Clustal X program (26), and the amino acids were deduced by using the MegAlign program. On the basis of the aligned sequences, phylogenetic trees were constructed by the neighbor-joining method (21) and the parsimony methods in the PAUP package (25). The \( rpoB \) sequence of \( H. \) campbellii was determined simultaneously and was used as an outgroup. Bootstrap values were evaluated from 1,000 replicate datasets. Homoplasy tests (18) were performed by using the HOMOPLASY program, and split decomposition was analyzed by using the SPLITSTREE program (version 3.1; http://www.mlst.net).

**Typing of \( H. \) pylori from gastric biopsy specimens.** By using computer-aided analysis (MapDraw package, Windows version 3.12e; DNASTAR) of the \( rpoB \) DNA sequences determined as described above, BsmFI was found to distinguish two \( H. \) pylori types. Gastric biopsy specimens were obtained from the antrums of 200 \( H. \) pylori patients at gastroscopy. The samples were separately used for genotyping by PCR restriction analysis. \( H. \) pylori was primarily detected and identified by the rapid urease test, \( rpoB \) PCR restriction analysis, and \( glmM \) PCR (16). DNAs were extracted from the biopsy specimens as described above and used for \( rpoB \) typing. Ten microtiter plates of the \( rpoB \) PCR products was transferred to a fresh microcentrifuge tube and digested with BsmFI (R0572S; New England Biolabs, Beverly, Mass.), according to the supplier's instruction. 

**IL-8 assay.** Interleukin-8 (IL-8) levels in the culture supernatants were measured as described previously (33). Briefly, MKN45 cells were grown to preconfluent monolayers (5 \( \times \) 10\(^5\) cells/ml) and then plated into 24-well plates (1 \( \times \) 10\(^5\) cells/well) and cultured for 2 days (5 \( \times \) 10\(^5\) cells/ml for each well). Twenty strains of each type of \( H. \) pylori isolated from Korean patients were cultured on brucella agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing 10% fetal calf serum in a microaerobic atmosphere at 37\(^\circ\)C for 48 h and added to the MKN45 cells (ratio of \( H. \) pylori cells to MKN45 cells, 100:1), and the mixture was then incubated at 37\(^\circ\)C for 20 h in 5% CO\(_2\). IL-8 levels were then measured by using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn.), as instructed by the manufacturer. Values were plotted by using the Box plot program (SigmaPlot 2000 for Windows, version 6.00; SPSS Inc., Chicago, Ill.) to show median values and confidence intervals. Statistical analysis of the IL-8 assay results was performed by the Mann-Whitney rank sum test. A \( P \) value of less than 0.05 was accepted as statistically significant.

**Nucleotide sequence accession numbers.** The \( rpoB \) sequences of \( H. \) pylori strains 26695 and J99 were retrieved from GenBank (accession nos. AE000625 and AE001540, respectively).

**RESULTS**

**\( rpoB \) genotype.** \( rpoB \) DNA (458 bp) containing a highly conserved region was amplified (16) and sequenced from 535 clinical \( H. \) pylori isolates obtained from 12 countries. Because many countries were represented by small numbers of isolates and, thus, may not represent the predominant strains of the particular geographical areas, we grouped strains by large geographical regions (Table 1). The \( rpoB \) sequences (363 bp) determined were aligned for the Homoplasy test, split decomposition analysis, and phylogenetic study. The Homoplasy index of \( H. \) pylori was found to be 0.519, which is higher than those reported for other bacteria (18, 23). This suggests frequent interstrain recombinations among the \( H. \) pylori population. Split decomposition analysis of \( rpoB \) showed a network topology and star phylogeny (data not shown), which are consistent with a recombinational population structure.

Another interesting finding was that although it was not robustly supported by bootstrap values, the \( H. \) pylori population could be separated into two major groups by nucleotide sequence analysis. In accord with previous reports on genotype analysis (1, 5, 8, 12, 13, 29, 32), the geographical distributions of these two groups were in agreement with their phylogenetic relationships. One group was termed the Asian group, and the other was termed the non-Asian group, which was mostly composed of Western \( H. \) pylori strains (North and South American, European, and South African strains) and included strains 26695 and J99 (Fig. 1A). Although marked genetic heterogeneity was observed, the clustering of \( H. \) pylori strains into...
FIG. 1. Phylogenetic relationships of 100 H. pylori isolates inferred from partial rpoB DNA sequences (A) and RpoB amino acid sequences (B). The H. pylori population was separated into an Asian group, to which most of the Asian strains belonged, and a non-Asian group, which was mainly composed of Western strains (North and South American, European, and South African strains), including strains H. pylori 26695 and H. pylori J99, by nucleotide sequence analysis (A). Two large groups (RpoBAla and RpoBThr) in the amino acid tree (B) were attributed to the identity of the 497th residue of each strain, which is either alanine or threonine. RpoBThr strains have the suffix T. The tree was constructed by the neighbor-joining method in the PAUP package. The bootstrap values presented at the corresponding branches were evaluated from 1,000 replications, and values less than 50% are not indicated.
different groups by geographical regions by rpoB analysis was also compatible with the findings of other studies (5, 17, 29, 31).

Deduced amino acid. For protein-coding genes, phylogenetic relationships based on amino acid sequences are often more significant than those based on nucleotide sequences (8, 20). Population genetics data based on nucleotide sequences are often inadequate for the study of protein-coding genes because variations are usually found at the third bases of codons (the wobble position), and these variations do not af-
Of the 200 Korean biopsy specimens, 129 strains (32.4%) were of the RpoB Ala type. Interestingly, almost all H. pylori strains (133 strains [66.2%]) from Western countries, including H. pylori 26695 and J99, whose sequences were retrieved from GenBank, were of the RpoBAla type. Only three Colombian strains and one North American (U.S.) strain (2.9%) were of the RpoBThr type. The amounts of IL-8 secreted by MKN45 cells infected with RpoB Thr strains were significantly higher (P < 0.05) than the amounts secreted by cells infected with RpoBAla strains (Fig. 3).

![Fig. 3. Secretion of IL-8 from MKN45 cells cocultured with the two different H. pylori strains, RpoBThr (n = 20) and RpoBAla (n = 20). All the H. pylori strains were cagA positive (East Asian-type CagA). The amounts of IL-8 protein excreted by the cells was measured by enzyme-linked immunosorbent assay. The levels of IL-8 secreted by RpoBThr-type infected cells were found to be significantly higher (P < 0.05 by the Mann-Whitney rank sum test) than the levels secreted by RpoBAla-type infected cells.](http://jcm.asm.org/)

### Table 2. Distributions of the 394 East Asian patients from whom the H. pylori strains were isolated

<table>
<thead>
<tr>
<th>Clinical status</th>
<th>RpoBThr type</th>
<th>RpoBAla type</th>
<th>Subtotal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric cancer</td>
<td>88 (66.2)</td>
<td>45 (33.8)</td>
<td>133</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>24 (75.0)</td>
<td>8 (25.0)</td>
<td>32</td>
</tr>
<tr>
<td>Gastritis</td>
<td>59 (66.3)</td>
<td>30 (33.7)</td>
<td>89</td>
</tr>
<tr>
<td>Benign gastric ulcer</td>
<td>17 (56.7)</td>
<td>13 (43.3)</td>
<td>30</td>
</tr>
<tr>
<td>Lymphoid hyperplasia</td>
<td>2 (100)</td>
<td>0 (0.0)</td>
<td>2</td>
</tr>
<tr>
<td>Within normal limit</td>
<td>3 (75.0)</td>
<td>1 (25.0)</td>
<td>4</td>
</tr>
<tr>
<td>Unknown</td>
<td>70 (67.3)</td>
<td>34 (32.7)</td>
<td>104</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td>263 (66.8)</td>
<td>131 (33.2)</td>
<td></td>
</tr>
</tbody>
</table>

* a Korea, Japan, Hong Kong, and Taiwan.
* b No pathological finding was observed.

Infect the amino acid sequence and thus result in synonymous substitutions (3, 8). Our analysis of the amino acid sequence of *rpoB* shows that the *H. pylori* strains could also be separated into another two large groups on the tree based on the amino acid sequences (Fig. 1B), and this was attributed to the identity of the 497th residue of each group, which was either alanine (GCT) or threonine (ACT). These groups were designated into another two large groups on the tree based on the amino acid sequences. Only the PCR product of RpoBThr was digested (lanes 4 and 5 [248 and 210 bp]) and lanes 6 and 7 [248, 116, and 94 bp]), while DNAs from the RpoBAla types were not (lanes 1 to 3 [458 bp]). Lane M, size marker (6xX74 replicative-form DNA digested with HaeIII). The numbers next to the gels are in base pairs.

**FIG. 2.** Differentiation of RpoBThr and RpoBAla type *H. pylori* strains by PCR-restriction fragment length polymorphism analysis (with BsmFI) of *rpoB* DNA. Amplified *rpoB* DNAs (458 bp) of *H. pylori* were digested with BsmFI and electrophoresed in a 3% agarose gel. DNAs from the RpoBThr types were digested (lanes 4 and 5 [248 and 210 bp] and lanes 6 and 7 [248, 116, and 94 bp]), while DNAs from the RpoBAla types were not (lanes 1 to 3 [458 bp]). Lane M, size marker (6xX74 replicative-form DNA digested with HaeIII). The numbers next to the gels are in base pairs.

**FIG. 3.** Secretion of IL-8 from MKN45 cells cocultured with the two different *H. pylori* types, RpoBThr (n = 20) and RpoBAla (n = 20). All the *H. pylori* strains were cagA positive (East Asian-type CagA). The amounts of IL-8 protein excreted by the cells was measured by enzyme-linked immunosorbent assay. The levels of IL-8 secreted by RpoBThr-type infected cells were found to be significantly higher (P < 0.05 by the Mann-Whitney rank sum test) than the levels secreted by RpoBAla-type infected cells.
DISCUSSION

_H. pylori_ infection is a major cause of gastritis and is considered an important risk factor for stomach cancer. However, the prevalence of _H. pylori_ alone does not sufficiently explain the striking difference in the geographical incidences of gastric cancer. While the association of the _cag_ pathogenicity islands with an increased risk of gastric cancer was proved, it cannot explain the differences in disease presentations caused by the different _cagA_ _H. pylori_ types in different geographical areas, namely, Eastern and Western countries. Furthermore, in Korea and Japan essentially all isolates are positive for the _cagA_ type that encodes the East Asian _cagA_ type. Thus, to test the hypothesis that a more virulent _H. pylori_ population exists in East Asia, we analyzed _H. pylori_ isolates on the basis of the sequence of the protein-coding gene, _rpoB_, which encodes the β-subunit of DNA-dependent RNA polymerase.

DNA-dependent RNA polymerase is a principal enzyme in the transcriptional process and of many regulatory pathways that control gene expression in living organisms. It is evolutionarily conserved in sequence, structure, and function from bacteria to humans (19, 24, 34). A high level of genetic diversity among _H. pylori_ strains has also been observed by 16S ribosomal DNA sequence analysis (27). However, as a protein-coding gene, _rpoB_ provided several advantages over 16S ribosomal DNA for phylogenetic analysis, which offered only a moderate power to discriminate or distinguish between species and strains (14, 15, 19). With _rpoB_ the analysis can be performed at both the nucleotide and the amino acid sequence levels and the _H. pylori_ strains can be grouped according to both the amino acid and the nucleotide sequences. The amino acid sequence-based grouping of _H. pylori_ led to the discovery of a novel _RpoB_ polymorphism (RpoB<sup>Thr</sup>-RpoB<sup>Ala</sup>) at residue 497. The prevalence of the RpoB<sup>Thr</sup> type was notable only in isolates from East and Southeast Asia. However, because the _H. pylori_ strains used for _rpoB_ analysis were collected retrospectively and the clinical information for many patients was unknown, we are very cautious not to conclude that a correlation between an _RpoB_ type with a certain gastrointestinal disease group exists.

Gastric cancer is generally thought to arise through a series of mucosal changes leading to atrophic gastritis caused by _chronic H. pylori_ infection. Chronic _H. pylori_ infection causes abnormal changes in the gastric mucosa, such as severe infiltration of the lamina propria by polymorphonuclear and mononuclear cells, and increases in epithelial cell proliferation, resulting in atrophic gastritis and focal intestinal metaplasia in an animal model (11). The proinflammatory chemokine IL-8 plays an important role in _H. pylori_-related inflammation by recruiting neutrophils and lymphocytes into the gastric mucosa (2, 4). We measured the IL-8 levels in cultured MKN45 cells after _H. pylori_ infection. Of interest, strains polymorphic at the 497th residue induced different amounts of IL-8 secretion, with strains with the RpoB<sup>Thr</sup> type inducing more IL-8 secretion than those with the RpoB<sup>Ala</sup> type. This difference in levels of IL-8 secretion did not correlate with the _recA_ group (group I and group II), as defined in a previous report (17; data not shown).

These data suggest that additional factors are responsible for enhanced virulence among _H. pylori_ strains and may provide important clues to the question of why the incidence of _H. pylori_-induced clinical disease differs so markedly between East Asia and the West. While it is clear that the presence of the East Asian-type _cagA_ cannot be solely credited with this difference in IL-8 induction, the specificity of the RpoB polymorphism in IL-8 induction have yet to be explored. The RpoB polymorphism may affect the function of RNA polymerase. Although the presence of the RpoB polymorphisms correlated with the geographical locations of the isolates and with the levels of IL-8 induction in vitro, it did not correlate with clinical presentation. The question remains whether _RpoB_ polymorphisms are directly involved in clinical outcomes or whether they are actually a marker linked to another factor responsible for increased virulence, independent of the polymorphism. Studies are planned to test the effects of the Thr→Ala substitution on IL-8 secretion in vitro to investigate whether the polymorphism is directly related to the induction of enhanced IL-8 secretion.

In conclusion, this _H. pylori_ population study based on _rpoB_ nucleotide sequences, analysis of their deduced amino acid sequences, and the IL-8 assay provides evidence that a polymorphism in _RpoB_ may be related to the pathogenesis of _H. pylori_-associated gastric diseases. Because of different host-parasite interactions, we suggest that the classification of _H. pylori_ strains according to the _RpoB_ polymorphism should be an integral part of the study of _H. pylori_-mediated pathogenesis.

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