The Evaluation of Putative Virulence Factors of *Helicobacter pylori* for Gastroduodenal Disease by Use of a Short-Term Mongolian Gerbil Infection Model

Masao Akanuma, Shin Maeda, Keiji Ogura, Yuzo Mitsuno, Yoshihiro Hirata, Tsuneo Ikenoue, Motoyuki Otsuka, Takeshi Watanabe, Yutaka Yamaji, Haruhiko Yoshida, Takao Kawabe, Yuzo Mitsuno, Yoshihiro Hirata, Tsuneo Ikenoue, Masao Akanuma, Shin Maeda, Keiji Ogura

Few virulence determinants of *Helicobacter pylori* have been tested in vivo. We conducted this study to establish an animal model for their screening. Six-week-old male Mongolian gerbils were inoculated with wild-type *H. pylori* (TN2) or its isogenic mutant with deletion of *cagE* (TN2Δ*cagE*), total *cag* pathogenicity island (TN2Δ*cag* PAI), HP0499 (TN2ΔHP499), or HP0638 (TN2ΔHP638) (*n* = 5 each). The animals were killed 3 weeks later, and the density of bacteria and the degree of inflammation in the stomach were compared. Infection was established in all animals except those inoculated with TN2ΔHP638. TN2 and TN2ΔHP499, but not TN2Δ*cagE* and TN2Δ*cag* PAI, induced intense inflammation, although the densities of bacteria were similar. The Mongolian gerbil model was useful for the screening of virulence determinants in vivo, which confirmed the importance of *cag* PAI while questioning that of HP0499.

*Helicobacter pylori* is a gram-negative, spiral-shaped, microaerophilic bacterium that infects human gastric mucosa. The prevalence of its infection is very high in various countries, including Japan, and the bacterium is now recognized as a major cause of chronic active gastritis, peptic ulcers, and gastric malignancies [1–8]. However, clinical manifestation of *H. pylori* infection varies widely in each patient, and most patients are virtually asymptomatic. This pleomorphism is supposed to be caused, in part, by strain diversity, and various virulence determinant factors have been proposed.

Among them, the *cag* pathogenicity island (*cag* PAI), a 40-kb cluster of ~30 genes of supposedly extraneous origin, is the most extensively investigated. Epidemiological studies have shown an association between advanced gastroduodenal diseases and infection with *cag* PAI–positive strains [9, 10]. We and others have shown that the presence of intact *cag* PAI is essential for NF-κB activation and interleukin (IL)–8 induction in human cells in vitro [11–23]. Moreover, by using *cagE* (a gene in *cag* PAI) knockout mutant *H. pylori*, we showed recently that *cag* PAI plays an essential role in inflammatory reaction and ulcer formation in Mongolian gerbils in vivo [24]. Recent studies have demonstrated that *cagE* and other genes in *cag* PAI code a type IV secretion system, which translocates the CagA protein inside the gastric epithelial cells [25, 26]. In contrast, deletion of *vacA*, another putative virulence determinant indicated by some studies in vitro, did not affect pathogenicity in Mongolian gerbils, rendering its clinical importance doubtful [24].

Several other genes outside *cag* PAI also were indicated as possible virulence determinants. HP0499, a homologue of *E. coli* *pldA*, recently was reported to code a phospholipase and to play a role in the colonization of the gastric mucosa [27]. Another gene, HP0638, or *oipA*, which codes 1 of the 32 outer membrane proteins, recently was reported to be associated with IL-8 induction in vitro [28]. However, the importance of these genes in pathogenesis in vivo has not been examined.

*H. pylori* is a human-specific pathogen and does not cause intense inflammation in conventional experimental animals. The Mongolian gerbil is an exception: recent studies have indicated that ulcers, intestinal metaplasia, and even adenocarcinoma develop during long-term *H. pylori* infection in the animal [29–31]. The gerbil model may be valuable not only in elucidating *H. pylori*–induced neoplasia but also in evaluating virulence factors in vivo, in which a shorter-term model will be preferable. In the present study, we used a 3-week Mongolian gerbil model to examine 2 recently proposed virulence genes, HP0499 and HP0638, as well as *cag* PAI, for their role in inflammation in vivo.
Materials and Methods

Bacterial strains and culture conditions. Clinical isolates of *H. pylori* (TN2), its isogenic mutants (TN2ΔcagE, TN2ΔHP499, and TN2ΔHP638), and cag PAI totally deleted mutant (TN2Δcag PAI), were used in this study. After 1 passage in vitro, all strains except TN2ΔHP638, which did not colonize in the animal, were used after 1 passage in Mongolian gerbils. TN2—which is positive for cag PAI, HP0499, and HP0638—shares an ancestral strain with TN2GF4, which induced gastric cancer in Mongolian gerbils in a study published elsewhere [30]. *H. pylori* cells were cultured in Brucella broth culture medium (Becton Dickinson) that contained 2.5% fetal bovine serum (Crasera International) and Glaxo selective supplement A (10 mg/L vancomycin, 3.3 mg/L polymyxin B, 20 mg/L bacitracin, 10.7 mg/L nalidixic acid, and 5 mg/L amphotericin B) in a microaerophilic atmosphere generated by CampyPak-Plus (Becton Dickinson) at 37°C for 24 h. Cultures were supplemented with sterilized glycerol at a final concentration of 15% and were stored at −80°C until use.

Construction of the mutants. The *cagE* mutant strain of TN2 (TN2ΔcagE) was constructed as described elsewhere [20], and disruption of the *cagE* gene was confirmed by Southern hybridization and polymerase chain reaction (PCR) that used primers HPPB-1F (TCTATAAAGAGAGGGGTGTT) and HPPB-1R (GGCTAATC

...TTTTGCTCTT) and 499-R (TGCCCTAGCCCTCTTTATCG) or use of the long PCR method with primers 499-F (ATGAAAAGCATAAGCAATTGTTCTGTCTTTAGC) and then was inserted at KpnI site of pCR/A::km, which resulted in pCR/A. Then, a 1240-bp kanamycin resistance gene (km r) cassette at the nucleotide position SpI site, which resulted in pCR/HP499::cp and pCR/HP638::cp. DNA from plasmid pCR/HP499::cp or pCR/HP638::cp was transfected into TN2 strain by electroporation [32]. The bacteria were recovered, were plated onto nonselective plates, were incubated for 48 h, and were transferred onto agar plates with chloramphenicol. Chloramphenicol-resistant *H. pylori* colonies were selected and screened for the allelic exchange by PCR and were confirmed as having the disrupted HP0499 or HP0638, respectively. HP0499 and HP0638 each was disrupted by insertion of a chloramphenicol resistance gene (cp) cassette at the nucleotide position SpI site, which resulted in pCR/HP499::cp and pCR/HP638::cp. DNA from plasmid pCR/HP499::cp or pCR/HP638::cp was transfected into TN2 strain by electroporation [32]. The bacteria were recovered, were plated onto nonselective plates, were incubated for 48 h, and were transferred onto agar plates with chloramphenicol. Chloramphenicol-resistant *H. pylori* colonies were selected and screened for the allelic exchange by PCR and were confirmed as having the disrupted HP0499 or HP0638 by Southern hybridization.

The *cag* PAI totally deleted mutant of TN2 was constructed by disruption of all genes between *cagA* and the most upstream portion of *cagII*. First, the most downstream 710-bp fragment of *cagA* was amplified by use of the long PCR method with primers 499-F (ATGAAAAGCACATTGTTCTGTCTTTAGC) and 499-R (TGCCCTAGCCCTCTTTATCG) or primers 638-F (ATGAAAAGCAATTGTTCTGTCTTT) and 638-R (GGCGTCTTTGATTGCTCAT). Each was cloned into the plasmid vector pCRII (Invitrogen), named pCR/PKO. DNA from plasmid pCR/PKO was transfected into the TN2 strain by electroporation [32]. The bacteria were recovered and plated onto nonselective plates and were incubated for 48 h. Then the bacteria were transfected onto agar plates with kanamycin. Kanamycin-resistant *H. pylori* colonies were selected and screened for the allelic exchange by the long PCR method with primers PAI K.O-F2 (GGATCCTCACTTAAACAGTACTA) and cagA-R (TCAAGATTTTTTGGAAACCCAC), both placed outside of primers cagII-F1 and cagA-13R.

Southern blot analysis. Genomic DNA was extracted from cultured mutant strains of TN2 with the SepaGene kit (Sankyo Junyaku), and 5 μg of each genomic DNA was digested with HindIII for 12 h at 37°C, was electrophoresed on 1% agarose gel, and was transferred to a nylon membrane (Amersham International), as described by Sambrook et al. [33] The DNA samples were hybridized to previously prepared DNA probes, which were digested from cloned plasmids and labeled with [α-32P]dCTP with the Ready To Go DNA labeling kit (Pharmacia Biotech). The membrane was pre-treated with ExpressHyb solution (Clontech) at 60°C for 1 h, and hybridization was carried out at 60°C for 2 h. The membrane was washed 4 times with 2× standard saline citrate (SSC)/0.05% SDS for 10 min at room temperature and twice with 0.1× SSC/0.1% SDS for 20 min at 50°C, and then autoradiography was performed by use of a BAS 2000 image analyzer (Fuji PHOTO Film).

Animals. Six-week-old male specific pathogen–free Mongolian gerbils (MON/Im/s/Gbs Slc) were purchased from Japan SLC and were maintained under standard laboratory conditions (room temperature, 23°C ± 2°C; relative humidity, 55% ± 5%; 12/12 h light/dark cycle) with free access to a commercial rodent diet (CE-2; Clea Japan) and tap water.

Five animals each were assigned to 5 groups and were inoculated with 1 mL culture broth either of wild-type TN2, TN2ΔcagE, TN2ΔHP499, TN2ΔHP638, or TN2Δcag PAI via intragastric gavage for fasting for 24 h. Each dose contained 5.5 ± 0.5 × 107 cfu of *H. pylori*. Other animals served as intact controls without challenge with bacteria. Animals were killed 3 weeks after inoculation, and their stomachs were divided into halves longitudinally. One half of each stomach was fixed in Carnoy’s fixative for histological examination, and the other half was used for quantitative determination of the bacterium by culture.

Culture study. The density of bacteria colonizing in the stomach was measured as described elsewhere [30]. In brief, half the excised stomach was homogenized with physiological saline, was inoculated immediately on Modified Skirrow’s agar, and was incubated for 4 days at 37°C under microaerobic conditions. Colonies were identified as *H. pylori* by morphology and urease activity. The number of colonies per plate was counted and expressed as the log colony-forming units per gastric wall. The stability of the mutation during infection was confirmed by genotyping by PCR and testing for kanamycin or chloramphenicol resistance of the recovered bacteria.

Histological examination. Another half of the stomach was stapled onto paper and was fixed in Carnoy’s fixative. The fixed gastric tissue was processed for histopathological examination, and paraffin-embedded sections were sliced and stained with hematoxylin-eosin or May-Grünwald-Giemsa. The grade of acute chronic gastritis was scored as 0, no gastritis; 1, mild gastritis (gastritis with mild neutrophil and mononuclear cell infiltration in some part of the pyloric region); 2, moderate gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastritis (gastritis with severe neutrophil and mononuclear cell infiltration in wide area of the pyloric region); and 3, severe gastris...
cell infiltration throughout the pyloric region and a part of the fundic region). The scores of chronic active gastritis were compared by the Mann-Whitney U test and Steel’s multiple comparison. The difference was considered to be significant when $P < .05$.

**IL-8 protein assay.** Gastric epithelial cells (AGS cell line; $1 \times 10^6$ cells/well) were cultured in Ham’s F12 medium with or without bacterial cells ($1 \times 10^6$ cfu/well) for 24 h at 37°C in a 95% air and 5% CO$_2$ humidified incubator. The culture medium was supplemented with 0.1% bovine serum albumin and 2 mM l-glutamine, without any antibacterial agents. At 18 h, supernatant was aspirated and frozen at -270°C until assayed. IL-8 protein levels in cell culture supernatant were determined by EIA after appropriate dilution. The EIA was carried out by use of Human Interleukin-8 Enzyme Immunoassay Kit (BioSource International), according to the manufacturer’s instructions.

**Results**

**Construction of the mutants.** The insertion of cp' into HP0499 or HP0638 of TN2ΔHP499 or TN2ΔHP638 was confirmed by Southern hybridization (figure 1). HP0499 of TN2ΔHP499 and HP0638 of TN2ΔHP638 were elongated because of chloramphenicol resistance gene insertion.

The construction of cag PAI totally deleted mutant of TN2 was confirmed by the long PCR method with primers PAI K.O-F2 and cagA-R (figure 2). In the cag PAI totally deleted mutants, the distance between these primers was 3520 bp and was able to be amplified by the long PCR method, but in the TN2 wild-type strain, the distance between the primers was too long (40 kb) to be amplified by the long PCR method. As the positive control, PCR amplification of the most upstream side of cagII with primers PAI K.O-F2 and cagII-R2 confirmed that the genomic DNA of 2 strains were extracted successfully.

**Colonization of wild type and mutants.** Three weeks after inoculation, *H. pylori* was recovered from 20 animals, except for 5 of 5 animals inoculated with TN2ΔHP638 and 5 of 5 intact controls. Bacteria density (mean ± SE log colony-forming units/gastric wall) was 5.92 ± 0.16, 6.02 ± 0.24, 5.87 ± 0.28, and 5.97 ± 0.39 ($P$, not significant), in the TN2, TN2ΔcagE, TN2ΔHP499, and TN2Δcag PAI groups, respectively (table 1). TN2ΔHP638 was inoculated to other 10 animals, but infection was established in none.

**Histological examination.** Figures 3 and 4 show the histopathologic changes in the gastric mucosa of Mongolian gerbils 3 weeks after *H. pylori* inoculation. The mean score of grade of inflammation in each group is summarized in figure 5. Gerbils infected with TN2ΔcagE or TN2Δcag PAI had significantly milder inflammation than those infected with the wild-type TN2 strain, as we previously noticed after a longer-term infection. However, infection of TN2ΔHP499 induced moderate to severe gastritis in all animals, comparable to that induced by wild-type infection. In these animals, dense neutrophil infiltration throughout from the

![Figure 1](image1.png)  
**Figure 1.** DNA samples of TN2, TN2ΔHP499, and TN2ΔHP638 hybridized with HP0499 (A) and HP0638 (B) probes. HP0499 of TN2ΔHP499 and HP0638 of TN2ΔHP638 were elongated because of chloramphenicol resistance gene insertion.

![Figure 2](image2.png)  
**Figure 2.** Polymerase chain reaction (PCR) amplification of whole cag pathogenicity island (cag PAI; lane 2, TN2; lane 3, TN2Δcag PAI) and the most upstream side of cagII (lane 4, TN2; lane 5, TN2Δcag PAI). The primers, PAI K.O-F2 and cagA-R, used for the whole cag PAI span (40 kb), did not amplify the cag PAI in TN2 (lane 2). In the TN2Δcag PAI, km’ replaced the cag PAI except at the end portions, which resulted in a 3520-bp PCR product (lane 3). The upstream 1210 bp was preserved in the TN2Δcag PAI and amplified by primers PAI K.O-F2 and cagII-R2 (lane 5). Lane 1, Marker lane.
depth to the outer layer of mucosa (figure 3A and 3B), and dense mononuclear cell infiltration in the lamina propria and submucosa were observed in a wide area of the pyloric region, where the normal mucosal architecture was almost lost and replaced with hyperplastic epithelium. (figure 4). In some cases (3/5 animals infected with TN2 and 2/5 animals infected with TN2ΔHP499), inflammatory cell infiltration was observed in the adjacent part of the fundic region. Animals infected with TN2ΔcagE and TN2Δcag PAI showed no gastritis or mild gastritis characterized by mild neutrophil and mononuclear cell infiltration in a small part of the pyloric region (figure 3C and 3D). No evidence of gastritis was found in any of the control animals (figure 3E).

**Effect of *H. pylori* strains on gastric epithelial IL-8 secretion.** The mean levels of IL-8 secreted by AGS cells over 24 h after stimulation by each *H. pylori* strain are shown in figure 6. In AGS cells, IL-8 secretion induced by TN2, TN2ΔHP499, or TN2ΔHP638 was greater than that induced by TN2ΔcagE or TN2Δcag PAI. There was no significant difference between IL-8 secretion induced by TN2ΔcagE or TN2Δcag PAI and baseline secretion without bacteria.

### Table 1. Bacterial counts within the gastric wall of Mongolian gerbils 3 weeks after oral challenge with *Helicobacter pylori.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Infection rate</th>
<th>Bacterial recovery, mean ± SE log cfu/stomach</th>
</tr>
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<tbody>
<tr>
<td>TN2 (wild type)</td>
<td>5</td>
<td>5.92 ± 0.16</td>
</tr>
<tr>
<td>TN2ΔcagE</td>
<td>5</td>
<td>6.02 ± 0.24</td>
</tr>
<tr>
<td>TN2ΔHP499</td>
<td>5</td>
<td>5.78 ± 0.28</td>
</tr>
<tr>
<td>TN2Δcag PAI</td>
<td>5</td>
<td>5.97 ± 0.39</td>
</tr>
<tr>
<td>TN2ΔHP638</td>
<td>15</td>
<td>0 (0) ND</td>
</tr>
<tr>
<td>Intact control</td>
<td>5</td>
<td>0 (0) ND</td>
</tr>
</tbody>
</table>

**Note.** ND, not detected; PAI, pathogenicity island.

Figure 3. The pyloric region of Mongolian gerbils 3 weeks after inoculation. The animals inoculated with TN2 (A) and TN2ΔHP499 (B) showed dense neutrophil cell infiltration throughout, from depth to the outer layer of mucosa. The animals inoculated with TN2ΔcagE (C) and TN2Δcag pathogenicity island (PAI; D) and intact controls (E) showed no gastritis. Hematoxylin-eosin original magnification, ×70.
Discussion

In a previous study, we examined the stomachs of gerbils 62 weeks after H. pylori inoculation, where gastric ulcer, intestinal metaplasia, and gastric cancer were found in the animals inoculated with the wild-type TN2 strain but not in those inoculated with the cagE-deleted mutant [24]. Although the observed gastric lesion was limited to gastritis in the present 3-week study, the degree of inflammation differed significantly between the animals challenged with TN2 and those challenged with the cagE-deleted mutant. Thus, the short-term model is sufficient for the screening of inflammatory virulence of H. pylori strains.

An animal model was proposed that used C57BL/6 mice, where inflammation was prominent 13 weeks but not 3 weeks after inoculation [34]. Thus, H. pylori–induced inflammation needs a shorter occult period in Mongolian gerbils than in mice. This is probably related to the difference in the speed of bacterial proliferation in the stomach: the bacterial density reached a plateau at 3 weeks and did not increase thereafter in Mongolian gerbils, whereas the density was smaller at 3 weeks than at 13 weeks in mice [34]. Acute inflammation may cause achlorhydria, which may result in the appearance of lower bowel flora in the stomach. However, we counted the bacterial number using H. pylori–selective medium. Furthermore, because the degree of inflammation differed between the inoculated strains, we can conclude that the inflammation was caused by H. pylori.

In this study, we prepared a mutant strain of TN2 (TN2Δcag PAI) whose cag PAI was totally deleted and showed that the effect of total cag PAI deletion and that of cagE deletion had similar effect on inflammation. Because the bacterial density of those mutants was similar to that of the wild type, the deletion did not change the ability of colonization or proliferation but directly ameliorated inflammatory reactions in the host cells. We observed similar results when we used clinical isolates whose cag PAI was partially or totally deleted (authors’ unpublished observation). These data indicate that intact cag PAI is directly involved in inducing inflammatory reaction in the host, which is compatible with our previous observation in vitro that intact cag PAI is a prerequisite for the activation of NF-κB and the mitogen-activated protein kinase cascade [20, 23].

In this study, we examined 2 recently reported putative virulence factors, HP0499 and HP0638. Although HP0499 has been reported to play a role in establishing colonization in the gastric mucosa of mice [27], we did not find any difference of bacterial density or grade of inflammation between gerbils challenged with the wild-type or the HP0499-deleted strain. In addition, the wild-type strain and HP0499-deleted mutant similarly induced IL-8 secretion from gastric cancer cells. The discrepancy is possibly

![Figure 4](image_url)

**Figure 4.** Low magnification showing the hyperplastic epithelium and dense mononuclear cell infiltration in the lamina propria and submucosa in wide area of the pyloric region of gerbils 3 weeks after inoculation with TN2 (A) and TN2ΔHP499 (B). Hematoxylin-eosin original magnification, ×35.

![Figure 5](image_url)

**Figure 5.** Inflammation score was compared between gerbils infected with TN2 wild type, TN2ΔHP499, TN2ΔcagE, and TN2Δcag pathogenicity island (PAI) and intact controls. *Mann-Whitney U test; **Steel’s multiple comparison.
Acknowledgments

We thank Masafumi Nakao, for advice about animal experiments, and Mitsuko Tsubouchi, for technical assistance.

References

21. Li SD, Kersulyte D, Lindley JJ, Neelam B, Berg DE, Crabtree JE. Multiple genes in the left half of the cag pathogenicity island of Helicobacter pylori

Figure 6. The levels of interleukin (IL)-8 secretion in gastric epithelial cells induced by TN2 wild type, TN2 ΔHP499, TN2 ΔHP638, TN2 ΔcagE, and TN2 Δcag pathogenicity island (PAI) and controls.