Virulence Factors of *Helicobacter pylori* Responsible for Gastric Diseases in Mongolian Gerbil

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Abstract

Helicobacter pylori infection induces various gastroduodenal diseases. We examined the role of two genes, *vacA* and *cagE*, in the gastric pathogenesis induced by *H. pylori* using a long-term (62 wk) animal model. Reportedly, both genes are associated with the virulence of *H. pylori*: *vacA* encodes vacuolating cytotoxin, and *cagE*, with other genes in the *cag* pathogenicity islands, encodes a type IV secretion system. Mongolian gerbils were challenged in this study by a wild-type TN2 strain and its isogenic mutants of *cagE* or *vacA*. The wild-type and *vacA* mutants induced severe gastritis, whereas *cagE* mutants induced far milder changes. Gastric ulcer was induced at the highest rate (22/23) by the wild-type TN2, followed by the *vacA* mutant (19/28). No ulcer was found in the gerbils infected with the *cagE* mutant (0/27) or in controls (0/27). Intestinal metaplasia was also found in the gerbils infected with the wild-type (14/23) or *vacA* mutant (15/28). Gastric cancer developed in one gerbil with wild-type infection and in one with *vacA* mutant infection. In conclusion, the knocking out of the *cagE* gene deprived wild-type *H. pylori* of the pathogenicity for gastritis and gastric ulcer, suggesting that the secretion system encoded by *cag* pathogenicity island genes plays an essential role.

Key words: gastritis • inflammation • mutagenesis • pathogenicity • animal experiments

Introduction

Helicobacter pylori is a gram-negative bacterium infecting human gastric mucosa with worldwide prevalence. This bacterium plays an important role in the pathogenesis of chronic gastritis, peptic ulcers, gastric adenocarcinomas, and gastric mucosa–associated lymphoid tissue lymphomas (1–4).

Several factors have been proposed as possible virulence determinants. Among them, the CagA protein, encoded by the *cagA* gene, is found in ~50–60% of isolates in Western countries and is reportedly associated with advanced gastroduodenal diseases (5–8). CagA-positive strains reportedly induce IL-8 secretion in gastric epithelial cells (9). Moreover, recent studies showed that the *cagA* gene is a member of the *cag* pathogenicity island (PAI),[1] a 40-kb region of supposedly extraneous origin (10). Isogenic mutant studies demonstrated that some *cag* PAI genes, including *cagE*, which was initially reported as *picB*, are essential for IL-8 induction and nuclear factor (NF)-κB activation (10–14). Recent studies indicated that *cagE* and several other genes of *cag* PAI code a type IV secretion system, which translocates CagA protein inside the gastric epithelial cells to be tyrosine phosphorylated by a yet unknown mechanism (15–17). Indeed, deletion of *cagE* was found to impair the translocation of CagA.

VacA is a polymorphic gene encoding vacuolating cytotoxin, and the strains producing active cytotoxin are usually *cagA* positive and toxigenic (18–21). The VacA protein causes vacuolation in cultured cells and is considered a cytotoxin (19). The strains that possess vacuolating activity are isolated from chronic gastritis and peptic ulcer patients at high frequency (22, 23). A recent study showed that VacA alters the intracellular trafficking of proteins, increases the permeability of polarized epithelial cells, inhibits the process of antigen presentation, forms anion-selective channels in

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[1]*Abbreviations used in this paper:* NF, nuclear factor; PAI, pathogenicity island; RT, reverse transcription; WT, wild-type.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/2000/12/1601/09 $5.00
Volume 192, Number 11, December 4, 2000 1601–1609
http://www.jem.org/cgi/content/full/192/11/1601

lipid bilayers, and interferes with cytoskeleton-dependent cell functions (24). However, in spite of various studies on VacA in vitro and in vivo, the exact role of VacA in gastric pathogenesis in vivo remains to be understood.

Animals used for studying experimental *H. pylori* infection include monkeys, dogs, piglets, domestic cats, and rodents (25–31). Several of the described models are not optimal because the animals can not be handled with ease or in large numbers, germ-free conditions are necessary, and infection rates are low. Infection of conventional mice with *H. pylori* overcomes many of problems, although infection is not easily achieved, and the gastritis in mice induced by *H. pylori* is less intense than in humans (32). The Mongolian gerbil is an animal in which various gastrointestinal diseases such as gastritis, ulcers, intestinal metaplasia, and gastric cancer can be appropriately developed (33–35). We previously reported that TN2GF4, which shares an ancestral strain with TN2, the wild-type (WT) strain used in this study, induced gastric cancer in gerbils without using adjuvant chemical carcinogens (34). It was also reported that isogenic mutants of *vacA* and *cagA* colonized well, allowing for examination of the role of virulence markers on gastric pathogenesis in Mongolian gerbils (36, 37). We conducted this study to examine the pathological consequences of long-term infection with WT and mutant (Δ*cagE*, Δ*vacA*) *H. pylori* strains using specific pathogen–free Mongolian gerbils to evaluate the role played by those genes in gastric pathogenesis.

Materials and Methods

Bacterial Strains and Culture Conditions. An *H. pylori* clinical isolate (TN2) and its two isogenic mutants (TN2Δ*cagE* and TN2Δ*vacA*) were used in this study. TN2, a strain positive for the vacuolating cytotoxin and *cag* PAI, shares an ancestral strain with TN2GF4, which we previously reported to induce gastric cancer in Mongolian gerbils (34). *H. pylori* cells were cultured in Brucella broth culture medium (Becton Dickinson) containing 2.5% FBS (Cansera International, Inc.) and Glaxo selective supplement A (10 mg/liter vancomycin, 3.3 mg/liter polymyxin B, 20 mg/liter bacitracin, 10.7 mg/liter nalidixic acid, and 5 mg/liter 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 Isogenic cagE-negative Mutant (TN2ΔcagE) and vacA-negative Mutant (TN2ΔvacA). The *cagE* mutant strain of TN2 was constructed as described previously (13). In brief, *cagE* gene was amplified using the long PCR method with primers HPPB-1F and HPPB-1R and then cloned into the plasmid vector pCRII (Invitrogen), named pCR/CE. The cloned *cagE* gene was disrupted by insertion of a kanamycin resistance gene (km^r) cassette at nucleotide position 212, which resulted in pCR/CE::km. DNA from plasmid pCR/CE::km was transferred into the TN2 strain by electroporation. The bacteria were recovered and plated onto nonselective plates and incubated for 24 h. Then, the bacteria were transferred onto agar plates with kanamycin. Kanamycin-resistant *H. pylori* colonies were screened for the allelic exchange by PCR, and the disruption in *cagE* gene was confirmed by Southern hybridization.

The *vacA* mutant of TN2 was also constructed as described (14), and disruption of the *vacA* gene was confirmed by means of Southern hybridization and PCR using primers vacA-C1 and vacA-C2 (21). A loss of vacuolation activity was checked by use of a vacuolating assay.

Animals. 5-wk-old male specific pathogen–free Mongolian gerbils (MON/Jms/Gbs Slc) were purchased from Japan SLC, Inc. and maintained under standard laboratory conditions (room temperature, 23 ± 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. All experimental protocols described were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals in the Pharmaceutical Research Division of Takeda Chemical Industries, Ltd. and approved by the Ethical Committee specializing in animal experiments for our division.

Adaptation for Infection. Six animals were inoculated with broth culture containing $5.4 \pm 0.7 \times 10^7$ CFUs of TN2 (WT), TN2Δ*cagE*, or TN2Δ*vacA* via intragastric gavage after fasting for 24 h. Animals were killed 2–3 wk after inoculation, and stomachs were used for quantitative culture. New gerbils were inoculated with recovered bacteria, and infecting bacteria were recovered 3 wk after inoculation. These steps were repeated three times, and the efficiency of infection was assessed at each stage.

Long-Term Infection Model. 125 animals were divided into four groups. WT groups consisted of 31 animals that were inoculated with 1 ml of *H. pylori* TN2 broth culture via intragastric gavage after fasting for 24 h. Both the Δ*cagE* and Δ*vacA* groups, each consisting of 31 animals, were challenged with TN2Δ*cagE* or TN2Δ*vacA*, respectively. Each inoculated broth culture contained $5.5 \pm 0.5 \times 10^7$ CFUs of bacteria. 32 animals served as intact controls without the challenge of bacterium. The body weight of animals was checked every 3 mo. Animals were killed 62 wk after inoculation, and stomachs were divided into halves. One half 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 number of the infecting bacteria in the stomach was measured as previously described (34). In brief, after the stomach was opened along the greater curvature, the longitudinal half was homogenized with physiological saline. Modified Skirrow's agar was inoculated with a diluted aliquot and incubated for 4 d 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 calculated as the log CFU per gastric wall. Genotyping by PCR and testing for kanamycin resistance of the recovered bacteria from three animals of each group were performed to confirm the stability of the mutation during infection.

Histological Examination. The half of the stomach to be used for histopathological examination was stapled onto paper and fixed in Carnoy's fixative. Fixed gastric tissue was cut into longitudinal strips for histopathological examination. After processing for histology by routine methods, paraffin-embedded sections were cut and stained with hematoxylin and eosin (H&E) or with alcian blue, pH 2.5, periodic acid-Schiff (AB-PAS). Histopathological lesions of the glandular stomach were categorized as follows: (1) mild gastritis (gastritis with mild mononuclear cell infiltration and lymphoid follicle formation in the pyloric region); (2) severe gastritis (gastritis with dense neutrophil and mononuclear cell infiltrations, multiple lymphoid follicle formations, and hyperplastic epithelium throughout the pyloric region and a part of the fundic region); (3) ulcer; (4) intestinal metaplasia (diagnosed

by the presence of goblet cells positive for AB-PAS staining); (5) adenocarcinoma (diagnosed by the presence of atypical glands locally invading the muscle layer and destroying the original architecture); and (6) carcinoid (diagnosed by neuroendocrine nests greater than three glands in diameter).

DNA Preparation and PCR. Chromosomal DNA was isolated using SepaGene (Sanko Junyaku) from cultures of TN2, TN2Δ*cag*E, and TN2Δ*vac*A before and after three passages through gerbils and cultures isolated from each three animals after long-term infection with TN2, TN2Δ*cag*E, or TN2Δ*vac*A. PCR was carried out in 50-μl volumes containing 100 ng of genomic DNA, 0.5 U of AmpliTaq DNA polymerase (Applied Biosystems) or LA Taq (Takara Shuzo), 25 pmol of each primer, and 10 nmol of each deoxynucleoside triphosphate in a standard buffer. Cycling conditions were 30 cycles at 94°C for 15 s, 50°C for 15 s, and 72°C for a time dependent on the expected product size (1 min per kilobase). The PCR primers used in this study are shown in Table I (38).

Reverse Transcription–PCR. RNA was prepared from 24-h broth cultures of WT or mutant *H. pylori* strains using ISOGEN (NipponGene). A 10-μg sample of each RNA was treated with RNase-free DNase I (Roche Diagnostics) to remove contaminating DNA. We next prepared cDNA from a 2-μg aliquot of the DNase-treated RNA samples by use of Super Script II RNase H^- Reverse Transcriptase (GIBCO BRL) and random hexamer oligonucleotide primers. The reaction mixtures were diluted to 20 μl with H_2O, and 1 μl of each was used as a template for PCR with oligonucleotide primers specific for *cagA*, *cagF*, *cagH*, or HP0888 (Table I). DNase-treated RNA samples were also diluted at the same concentration and used as a template for PCR to confirm that DNA was removed from the RNA samples.

Table I. *Primers Used in This Study*

Region	Primer	Nucleotide sequence
cagA	cagA-F	5′-AACAGGACAAGTAGCTAGCC*
	cagA-R	5′-GATAACAGGCAAGCTTTTGA*
cagE	HPPB-1F	5′-TCTATAAAGAGAGGGGTGTT‡
	HPPB-1R	5′-GGCTAATCTTTGGTAATCAG‡
cagF	cagF-F	5′- ATGAAACAAATTTGCGTGA§
	cagF-R	5′-TCAATCGTTATTTTTGTTTT§
cagH	cagH-F	5′-ATGGCAGGTACACAAGCTAT§
	cagH-R	5′-TCACTTCACGATTATTTTAG§
cagM	cagM-F	5′-ATGCTTGCAAAAATTGTTTT*
	cagM-R	5′-CTATTCAAAGGGATTATTCT*
cagT	cagT-F	5′-CCATGTTTATACGCCTGTGT*
	cagT-R	5′-CATCACCACACCCTTTTGAT*
left end of cag	cagLEC-F	5′-ACATTTTGGCTAAATAAACGCTG*
	cagLEC-R1	5′-TCTCCATGTTGCCATTATGCT‖
vacA	vacA-C1	5′-ATGGAAATACAACAAACACACCGCA¶
	vacA-C2	5′-TTAGAAACTATACCTCATTCCTAAA¶
HP0887-0888	HP887F	5′-TCATAGCGATTGGCGTGGAA**
	HP888R	5′-GCTTGGAATTAGAGTCCTTA**
HP0888	HP888F	5′-ATGAACGCTGGAGTTTTACA**
	HP888R	5′-GCTTGGAATTAGAGTCCTTA**

*Reference 38.
‡Reference 13.
§Available from EMBL/GenBank/DDBJ under accession no. U60176.
‖Available from EMBL/GenBank/DDBJ under accession no. AC000108.
¶Reference 21.
**Available from EMBL/GenBank/DDBJ under accession no. AE000511.

Statistical Methods. The body weights of gerbils were compared by one-factor ANOVA (analysis of variance). The incidence of gastric lesion formation was compared by Fisher's exact probability test followed by the Holm's correction for multiple comparison. The differences were considered significant when the P value was <0.05.

Results

Colonization Efficiency of WT and Isogenic Mutant H. pylori Strains. To assess colonization ability in the gerbil's stomach, gerbils were inoculated with WT or mutants of *H. pylori* and killed 2–3 wk after inoculation, and the stomachs were then cultured for *H. pylori* (Table II). Recovered bacteria were cultured in vitro and then inoculated in other animals up to the third stage of in vivo passage. The strain TN2Δ*cagE* did not achieve an efficient rate of infection during the first stage, whereas WT and TN2Δ*vacA* infected the gerbils without failure. However, the rate of successful infection improved after in vivo passage. Those strains recovered after three passages were used for the 62-wk long-term infection study.

Long-Term Infection of WT and Mutant Strains. 31 animals in each group were inoculated with TN2, TN2Δ*vacA*, or TN2Δ*cagE*, and 32 animals served as the intact control group. During the 62 wk after inoculation, eight, four, three, and five animals died in the WT, Δ*cagE*, Δ*vacA*, and control groups, respectively. The mean body weight of surviving animals at 62 wk was 66 g in the WT, 77 g in the Δ*cagE*, 76 g in the Δ*vacA*, and 90 g in the control group (Fig. 1). Concerning mortality, there were no significant differences between groups other than the body weight curves, which were significantly lower in the WT than in the mutant and control group. Culture and histological examination were performed in 23, 27, 28, and 27 animals of each group (WT, Δ*cagE*, Δ*vacA*, and control, respectively; Table III). *H. pylori* was recovered from all animals in the challenged groups except for one animal in the WT group, and *H. pylori* was not cultured from animals in the control group. Bacteria density (log CFU per gastric wall) was (mean ± SE) 5.48 ± 0.28, 5.53 ± 0.15, 5.53 ± 0.20, and zero in the WT, Δ*cagE*, Δ*vacA*, and control groups, respectively (Table III).

Histological Examination. The histopathologic changes in the gastric mucosa of gerbils infected with *H. pylori* for

Table II. *Adaptation for Infection after Mutagenesis*

	1st stage	2nd stage	3rd stage
TN2 (WT)	6/6 (100)	5/5 (100)	5/5 (100)
TN2Δ*cagE*	2/6 (33)	5/5 (100)	5/5 (100)
TN2Δ*vacA*	6/6 (100)	5/5 (100)	5/5 (100)

Number of infected animals/total number of challenged animals (percentage with infection) are shown.

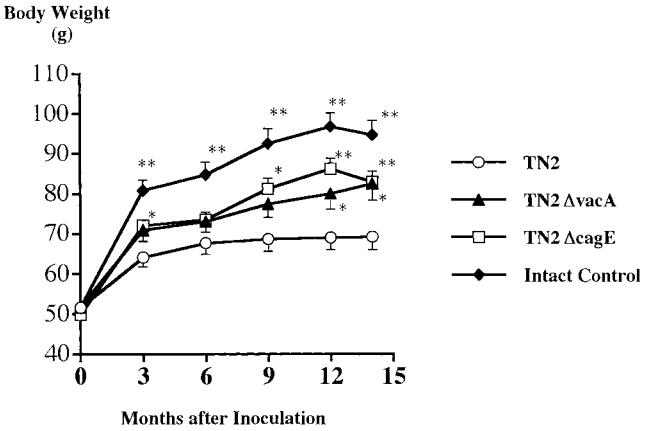

Figure 1. Changes in body weight of Mongolian gerbils inoculated orally with *H. pylori* TN2 (○) and its isogenic *vacA* (▲) or *cagE* (□) mutants and intact control gerbils (◆). The data and error bars represent means and the standard error of body weights.

62 wk are shown in Figs. 2–4 and summarized in Table IV. Inflammation was significantly reduced in the gerbils infected with TN2Δ*cagE* compared with those infected with TN2 or TN2Δ*vacA*. However, no significant differences were found in histopathological changes induced by TN2 or TN2Δ*vacA*. Severe gastritis was observed in all animals (100%) infected with TN2 and in 26 out of 28 animals (93%) infected with TN2Δ*vacA*. In these animals, dense infiltration of neutrophils and mononuclear cells and multiple lymphoid follicle formation were observed in the lamina propria and submucosa throughout the pyloric region as well as the adjacent part of the fundic region (Fig. 2, A, C, E, and G). The normal mucosal architecture was almost completely lost in these areas and replaced with hyperplastic epithelium. The mucosal layer was thickened remarkably, and pseudopyloric gland formation, cystic glandular dilatation, and invagination into the submucosa of hyperplastic glands were frequently noted. Animals infected with TN2Δ*cagE*, however, showed much milder gastritis (Fig. 2, B and F) restricted to the pyloric region and characterized by mild mononuclear cell infiltration with lymphoid follicle formation. Neutrophil activity and epithelial changes

Table III. *Bacterial Counts within the Gastric Wall of Mongolian Gerbils 62 wk after Oral Challenge of H. pylori*

Strain	Infection rate		Bacterial recovery
	n	Positive (%)	Log CFU per stomach*
TN2 (WT)	23	22 (96)	5.48 ± 0.28
TN2Δ*cagE*	27	27 (100)	5.53 ± 0.15
TN2Δ*vacA*	28	28 (100)	5.53 ± 0.20
Intact control	27	0 (0)	Not detected

*Mean ± SE.

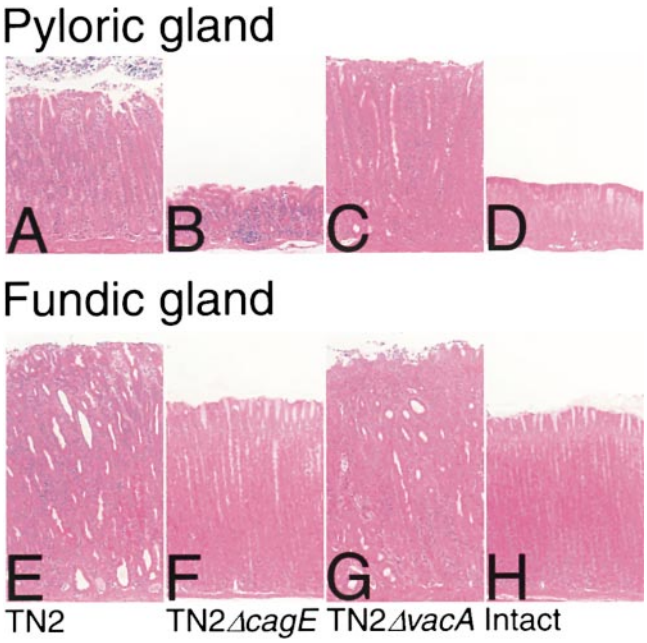

Figure 2. Histopathological findings in the gastric mucosa of gerbils 62 wk after *H. pylori* inoculation. Gastritis (A–D, pyloric region; and E–H, fundic region): severe gastritis characterized by dense neutrophil and mononuclear cell infiltrations and epithelial hyperplasia can be seen in the animals infected with TN2 (A and E) and TN2Δ*vacA* (C and G). The gastritis extends throughout the pyloric region (A and C) and into a part of the fundic region (E and G). Mucosal thickness increases remarkably as compared with uninfected animals (D and H). Only mononuclear cell infiltration and lymphoid follicle formation are noted in the pyloric region of animals infected with TN2Δ*cagE*, and neutrophil infiltration and epithelial alterations are not evident (B). There was no inflammation in the fundic regions of animals infected with TN2Δ*cagE* (F). H&E stain; original magnifications 35×.

were rarely seen. No evidence of gastritis was found in any of the control animals (Fig. 2, D and H).

Gastric ulcers were found in 22 out of 23 animals (96%) with TN2 and in 19 out of 28 animals (68%) with TN2Δ*vacA*. Ulcers were localized in the region of the fundus–pylorus border, and all ulcer lesions reached the submucosa or the proper muscular layer (Fig. 3, A and C). No gastric ulcers were found in animals with TN2Δ*cagE* or animals of the control group (Fig. 3, B and D). The prevalence of gastric ulcers was significantly higher in animals challenged with TN2 or TN2Δ*vacA* than in animals with TN2Δ*cagE* or in controls ($P < 0.0001$ for all pairs). In the Δ*vacA* group, the number of animals suffering from gastric ulcers was reduced significantly compared with the WT group ($P = 0.013$). Intestinal metaplasia, formed mainly near the ulcer (Fig. 4 B), was found in 14 out of 23 (61%), zero out of 27 (0%), 15 out of 28 (54%), and zero out of 27 (0%) animals in the WT, Δ*cagE*, Δ*vacA*, and control group, respectively.

An adenocarcinoma developed in two animals, one in the WT group and another in the Δ*vacA* group. Both tumors were located in the pyloric region, grew downward, and penetrated into the muscle layer (Fig. 4 A). The tumors retained a well-preserved glandular structure and con-

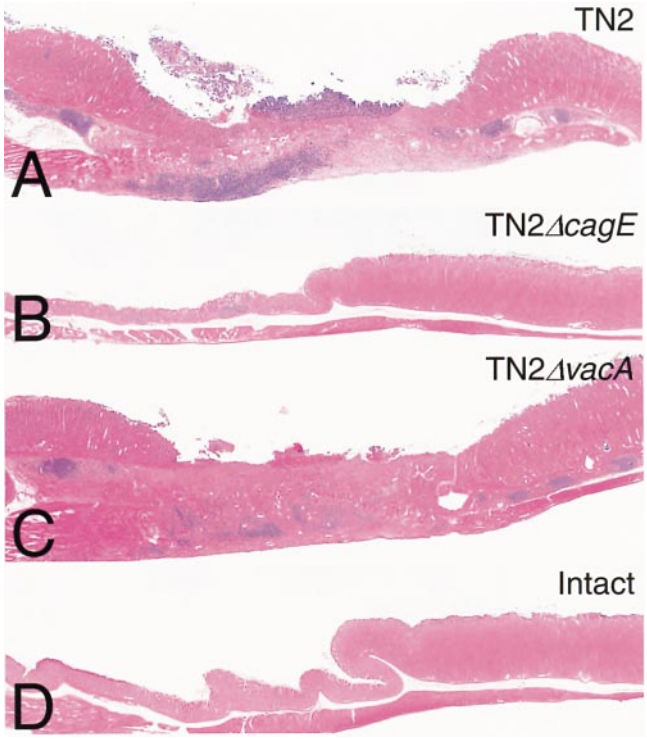

Figure 3. Ulcers, extending to the muscular layer, developed in the region of the fundus–pylorus border of animals infected with TN2 (A) or TN2Δ*vacA* (C). No gastric ulcers are seen in animals infected with TN2Δ*cagE* (B) or uninfected animals (D). H&E stain; original magnifications 10×.

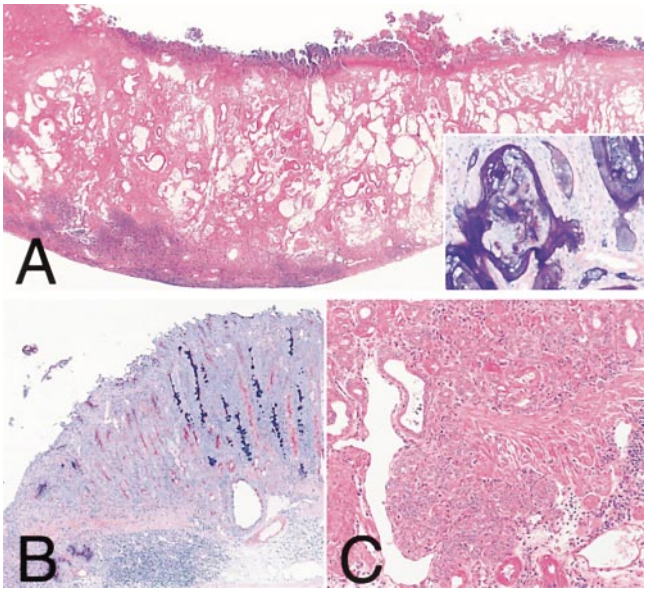

Figure 4. Other histopathological findings. Adenocarcinoma (A): an adenocarcinoma observed in animals infected with TN2. Neoplastic glands consisted of well-differentiated intestinal-type epithelium (inset) and penetrated into the muscle layer. Intestinal metaplasia (B): intestinal metaplasia observed in animals infected with TN2. Metaplastic epithelium containing AB-PAS–positive goblet cells frequently formed near the ulcer. Carcinoids (C): carcinoids detected in the animal infected with TN2Δ*vacA*. A and C, H&E stain; original magnifications 15 (A) and 80× (C). A, inset, and B, AB-PAS: original magnifications 80 (A, inset) and 30× (B).

sisted of well-differentiated columnar intestinal-type epithelium. In addition, carcinoids derived from the fundic mucosa were detected in one animal (4%) in the WT group and five animals (18%) in the Δ*vacA* group. These carcinoids were arranged in solid sheets or small nests in the deep lamina propria and occasionally spread into the submucosa (Fig. 4 C). Neither adenocarcinomas nor carcinoids were found in the gerbils in the Δ*cagE* group and control group. The incidence of gastric cancer and carcinoid did not differ significantly among the groups.

Genomic Stability of H. pylori through Infection Study. The stability of the *H. pylori* genome through in vivo passages was examined for the intended mutation and for the *cag* PAI. The disruption of the *cagE* and *vacA* genes in recovered bacteria from three animals of each infected group was confirmed as stable based on the size of the amplicons by PCR as compared with the original mutants. All recovered mutants were resistant to kanamycin, indicating that the kanamycin cassette still worked after 62 wk of infection (data not shown). The presence of five regions (*cagA*, *cagF*, *cagM*, *cagT*, and *cag*LEC) in *cag* PAI was examined by PCR. All cultures examined revealed positive amplification for the five regions (data not shown).

Detection of Gene Expression by Reverse Transcription–PCR. To investigate whether insertion of an antibiotic resistance cassette into *cagE* or *vacA* had polar effects on expression of the flanking genes located downstream of insertion, we performed reverse transcription (RT)-PCR on RNA samples prepared from WT strain (TN2), *cagE* mutant, and *vacA* mutant. Control PCR experiments verified that DNA was efficiently removed from RNA samples by DNase treatment. We then applied cDNA prepared from these samples to PCR experiments with oligonucleotide primers specific for the *cagA*, *cagF, cagH,* and HP0888 genes (Table I). A primer pair for the *cagA* gene, used as positive control of RT-PCR, produced amplicons of expected size in these strains to similar extent, indicating that cDNA was properly prepared from each sample. The RT-PCR product of the *cagF* or *cagH* gene was also amplified in TN2Δ*cagE* as well as in TN2, indicating that the km^r cassette insertion into *cagE* had no polar effect on transcription of *cagF* or *cagH*. This finding agrees with the previous report with Northern blot showing that *cagE* (*picB*) was cotranscribed with only *picA*, which is located upstream of *cagE* (12). Transcription of the HP0888 gene, which is located downstream of *vacA* (HP0887) in strain 26695, was also analyzed by RT-PCR (39). The primer pair encompassing the *vacA* gene and HP0888 amplified the expected product in the TN2 genome, indicating that the HP0888 gene is located downstream of *vacA* in TN2 genome as in that of the 26695 genome. RT-PCR analysis showed no difference in the transcription of HP0888 between TN2 and TN2Δ*vacA*, suggesting that the insertion of the km^r cassette into *vacA* gene did not affect the transcription of

Table IV. *Histological Findings in the Stomachs of Gerbils 62 wk after Oral Challenge of H. pylori*

Strain:	TN2 (WT)	TN2Δ*cagE*	TN2Δ*vacA*	Intact control
No. of animals:	23	27	28	27
Gastritis	23 (100)	27 (100)	28 (100)	0 (0)*
(Severe)	23 (100)	0 (0)*	26 (93)	0 (0)*
Gastric ulcer	22 (96)	0 (0)*	19 (68)**	0 (0)*
Intestinal metaplasia	14 (61)	0 (0)*	15 (54)	0 (0)*
Adenocarcinoma	1 (4)	0 (0)	1 (4)	0 (0)
Carcinoid	1 (4)	0 (0)	5 (18)	0 (0)

Rates are shown as percentages in parentheses. Significant differences against WT infection by means of Fisher's exact probability are indicated as the following: $*P < 0.0001$ and $**P = 0.013$.

HP0888. This result is consistent with the previous report that *vacA* gene is most likely transcribed alone (40).

Discussion

In this study, the role of virulence factors in the pathogenesis of *H. pylori* infection was investigated using Mongolian gerbils as an in vivo animal model. For this purpose, targeted genes were disrupted by homologous recombination, and the pathogenicity was compared in WT strains and isogenic mutants. All gerbils except one were infected with WT and mutant strains throughout this long-term study. Disruption of the genes did not affect the ability of *H. pylori* to colonize and replicate in the gerbil's stomach. Animals in every group survived for 62 wk, with only a few deaths, and the survival rate of each group did not differ significantly. However, gains in body weight differed significantly.

In humans, the effect of *H. pylori* infection on nutritional status of the host remains controversial. Reportedly, sustained *H. pylori* infection is associated with growth failure in Gambian infants (41). Fall et al. (42) also reported that in England, low weight at 1 y of age was associated with increased *H. pylori* seropositivity rates in males, although they did not decide whether *H. pylori* infection was the cause of growth failure or whether small infants were more susceptible to infection. On the other hand, there were reports from Bangladesh, Guatemala, and Nicaragua that found no association between *H. pylori* infection and malnutrition in children or infants (43–45). Quinonez et al. (44) concluded that the small differences in the nutritional status between the infected and uninfected children may be due to sociodemographic factors. The results of this study show that experimental infection with *H. pylori*, especially the WT, has led to a significant reduction in body weight 3 mo after inoculations. This infection model is purely experimental and, in design, is not affected by factors other than infection. Therefore, our observations indicate that infection with *H. pylori* may interfere with normal growth in Mongolian gerbils and further suggests that the same can occur in humans.

The colonization ability of *H. pylori* strains, especially TN2Δ*cagE*, was improved after the three passages through gerbils. It is known that the *cag* PAI often is lost during human infection, and it is discussed that strains that lost *cag* PAI may grow better than the *cag* PAI–positive parental strains in a subset of humans (46). To characterize the isolates obtained after the three passages through gerbils and the isolates after long-term infection in comparison with the pre-challenge strains, we examined the existence of genes in *cag* PAI by PCR. No major deletion of *cag* PAI was found, and it is possible that *cag* PAI does not affect adaptational capacity for infection in gerbils.

Deletions of the *cag* PAI and several *cag* insertion mutations reportedly block the induction of proinflammatory cytokine IL-8 in gastric epithelial cells in vitro (10, 13, 47). Recently, reports have noted the presence of *cag* PAI in association with *H. pylori*–mediated NF-κB activation in gastric epithelial cells, which is a critical regulator of the genes involved in inflammation, proliferation, and apoptosis (14, 48). The proteins encoded by genes located in *cag* PAI are suggested to function as secretion machinery exporting molecules possibly involved in *H. pylori*–host cell interaction (10, 49). In addition, CagA protein is noted to be translocated into gastric cells via *cag* PAI system and to be phosphorylated (15–17). The *cagE* gene is a homologue of the transporter component in *Agrobacterium tumefaciens* and *Bordetella pertussis* that engages in the transcellular transport of molecules (12). In this study, the role of the *cagE* gene product was assessed using gene disruption in an animal model suitable for the assessment of the pathogenesis of *H. pylori* infection. *CagE* knockout mutants, which possibly have a defect in the secretion apparatus, could not cause severe gastritis or induce the metaplastic changes often followed by the development of cancer (50). The WT and *vacA*-disrupted strains, however, induced severe gastritis and metaplasia in the gastric mucosa of gerbils, indicating the role of CagE protein in the pathogenesis of inflamed gastric mucosa. Moreover, it is tempting to speculate that the product of the *cagE* gene, one of the functional genes in *cag* PAI, may play a crucial role in the carcinogenesis that

accompanies *H. pylori* infection. Reportedly, other genes in *cag* PAI are also required in host–bacterial interactions in vitro; however, their in vivo role was not assessed in this study. Further investigations are necessary to elucidate the roles of other *cag* PAI genes in vivo.

The VacA protein has been regarded as a potential candidate responsible for the virulence of *H. pylori* as well as the cause of inflammation and ulcer formation in the stomach and duodenum (22–24, 51). However, as the VacA-positive strains often have intact *cag* PAI (52, 53), the responsibility of VacA in inducing gastritis and ulcer formation is still controversial. Oral administration of the purified cytotoxin caused ulceration in mice (51), and recent investigations revealed the pathogenic effect of VacA in epithelial cells. VacA inhibited the conversion of procathepsin D and the intracellular degeneration of epidermal growth factor (54) and also interfered with cytoskeleton-dependent cell functions and the transmission of signals related to cell spreading and growth (24). On the other hand, Ghiara et al. (55) suggest that VacA does not play an important role in eliciting inflammation using a mouse model with sonic extracts of *H. pylori*. Weel et al. (7) also reported that their analysis of clinical isolates showed an association of peptic ulcer disease with only CagA, not VacA.

In our study, Mongolian gerbils were challenged with the *vacA* knockout strain to assess the role played by vacuolating activity in pathogenesis in vivo. The *vacA*-disrupted strain caused inflammatory changes in the stomachs of gerbils to a degree similar to that in the WT, indicating that VacA or the vacuolating activity of bacteria is not essential for the inflammatory response of gastric mucosa. Gastric ulcer was induced in 96% of the animals infected with the WT strain, 68% of those infected with TN2Δ*vacA*, and in none of those infected with TN2Δ*cagE*. This result also indicates the important role, direct or indirect, of the *cagE* gene product in the pathogenesis of gastric ulcers. On the other hand, the disruption of *vacA* gene reduced the incidence of gastric ulcers significantly, although gastric ulcers were observed nevertheless in more than half of the animals in the Δ*vacA* group. These results suggest that *vacA* is not involved in the inflammation of gastric mucosa but may be partially responsible for gastric ulcer formation. However, there is a possibility that VacA is not directly involved in ulcerogenesis but still worsens gastric ulcer or delays its healing by degenerating epidermal growth factor (54) or interfering with cytoskeleton-dependent cell function (24).

In our previous study, 37% of gerbils contracted gastric cancer 62 wk after inoculation with the same WT *H. pylori* strain, TN2. In this study, two gerbils (one from the WT group and another from the Δ*vacA* group) developed gastric cancer, and none from the Δ*cagE* or control group did. Thus, the incidence of cancer development (4%) in this study was smaller than that reported in our previous study (34). Possibly, employment of a different breeder (Japan SLC, Inc.) of Mongolian gerbils (MON/Jms/Gbs Slc) in this study caused the difference in the incidence of cancer. Intestinal metaplasia also developed in fewer animals with WT TN2 (61%) than in our previous study (85%) when using the same time interval of 62 wk after inoculation. Therefore, a possible reason for the low incidence of cancer in this study may be the slow progression of chronic changes in the gastric mucosa as metaplasia due to genetic diversity of the host or unexpected differences in environmental factors. In fact, another long-term (~64 wk) experiment using 28 gerbils with TN2 infection confirmed the incidence of gastric adenocarcinoma as 21% (data not shown), with gerbils (MGS/Sea) purchased from the same breeder (Seiwa Experimental Animals) that was used in the previous study. Both breeders provided us with the descendants of 20 pairs of Mongolian gerbils captured at the Amur Basin in 1935. However, the ancestries of MGS/Sea diverged in 1973 and were separately inbred since that time, and those of MON/Jms/Gbs Slc diverged in 1979 and were inbred. Therefore, it is possible that there is a genetic diversity between the MGS/Sea and MON/Jms/Gbs Slc gerbils that may affect the inflammatory response as well as the process of carcinogenesis. Thus, it will be extremely interesting to further study the relation of gastric pathogenesis with genetic diversity of the host (Mongolian gerbil) and that of the infecting agent (*H. pylori*) and may eventually reveal the underlying mechanism of gastric carcinogenesis.

In conclusion, we showed in this study that *cagE* is responsible for various gastric lesions induced in the gerbils model, and in the future, this experimental animal model may help to elucidate the virulence factors responsible for gastric carcinogenesis. It has also been suggested that *cag* PAI may play an important role in the pathogenesis of gastric diseases related to *H. pylori* infection also in humans.

This study was supported in part by a Grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (11557040).

Submitted: 22 June 2000
Revised: 26 September 2000
Accepted: 6 October 2000

References

1. Graham, D.Y., G.M. Lew, P.D. Klein, D.G. Evans, D.J.J. Evans, Z.A. Saeed, and H.M. Malaty. 1992. Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. *Ann. Intern. Med.* 116:705–708.
2. Parsonnet, J., G.D. Friedman, D.P. Vandersteen, Y. Chang, J.H. Vogelman, N. Orentreich, and R.K. Sibley. 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* 325:1127–1131.
3. Nomura, A., G.N. Stemmermann, P.H. Chyou, I. Kato, P.G. Perez, and M.J. Blaser. 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. *N. Engl. J. Med.* 325:1132–1136.
4. Forman, D., D.G. Newell, F. Fullerton, J.W. Yarnell, A.R. Stacey, N. Wald, and F. Sitas. 1991. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *Br. Med. J.* 302:1302–1305.
5. Covacci, A., S. Censini, M. Bugnoli, R. Petracca, D. Bur-

roni, G. Macchia, A. Massone, E. Papini, Z. Xiang, N. Figura, et al. 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc. Natl. Acad. Sci. USA.* 90:5791–5795.

6. Tummuru, M.K., T.L. Cover, and M.J. Blaser. 1993. Cloning and expression of a high-molecular-mass major antigen of *Helicobacter pylori*: evidence of linkage to cytotoxin production. *Infect. Immun.* 61:1799–1809.
7. Weel, J.F., R.W. van der Hulst, Y. Gerrits, P. Roorda, M. Feller, J. Dankert, G.N. Tytgat, and A. van der Ende. 1996. The interrelationship between cytotoxin-associated gene A, vacuolating cytotoxin, and *Helicobacter pylori*-related diseases. *J. Infect. Dis.* 173:1171–1175.
8. Blaser, M.J., G.I. Perez-Perez, H. Kleanthous, T.L. Cover, R.M. Peek, P.H. Chyou, G.N. Stemmermann, and A. Nomura. 1995. Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer. Res.* 55:2111–2115.
9. Crabtree, J.E., and S.M. Farmery. 1995. *Helicobacter pylori* and gastric mucosal cytokines: evidence that CagA-positive strains are more virulent. *Lab. Invest.* 73:742–745.
10. Censini, S., C. Lange, Z. Xiang, J.E. Crabtree, P. Ghiara, M. Borodovsky, R. Rappuoli, and A. Covacci. 1996. *cag*, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. *Proc. Natl. Acad. Sci. USA.* 93:14648–14653.
11. Li, S.D., D. Kersulyte, I.J. Lindley, B. Neelam, D.E. Berg, and J.E. Crabtree. 1999. Multiple genes in the left half of the *cag* pathogenicity island of *Helicobacter pylori* are required for tyrosine kinase-dependent transcription of interleukin-8 in gastric epithelial cells. *Infect. Immun.* 67:3893–3899.
12. Tummuru, M., S. Sharma, and M. Blaser. 1995. *Helicobacter pylori picB*, a homologue of the *Bordetella pertussis* toxin secretion protein, is required for induction of IL-8 in gastric epithelial cells. *Mol. Microbiol.* 18:867–876.
13. Ogura, K., M. Takahashi, S. Maeda, T. Ikenoue, F. Kanai, H. Yoshida, Y. Shiratori, K. Mori, K.I. Mafune, and M. Omata. 1998. Interleukin-8 production in primary cultures of human gastric epithelial cells induced by *Helicobacter pylori*. *Dis. Dis. Sci.* 43:2738–2743.
14. Maeda, S., H. Yoshida, K. Ogura, Y. Mitsuno, Y. Hirata, Y. Yamaji, M. Akanuma, Y. Shiratori, and M. Omata. 2000. *Helicobacter pylori* activates NF-κB through a signaling pathway involving IκB kinases, NF-κB-inducing kinase, TRAF2, and TRAF6 in gastric cancer cells. *Gastroenterology.* 119:97–108.
15. Asahi, M., T. Azuma, S. Ito, Y. Ito, H. Suto, Y. Nagai, M. Tsubokawa, Y. Tohyama, S. Maeda, M. Omata, et al. 2000. *Helicobacter pylori* CagA protein can be tyrosine phosphorylated in gastric epithelial cells. *J. Exp. Med.* 191:593–602.
16. Stein, M., R. Rappuoli, and C. Covacci. 2000. Tyrosine phosphorylation of the *Helicobacter pylori* CagA antigen after *cag*-driven host cell translocation. *Proc. Natl. Acad. Sci. USA.* 97:1263–1268.
17. Odenbreit, S., J. Puls, B. Sedlmaier, E. Gerland, W. Fischer, and R. Haas. 2000. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science.* 287: 1497–1500.
18. Leunk, R.D., P.T. Johnson, B.C. David, W.G. Kraft, and D.R. Morgan. 1988. Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *J. Med. Microbiol.* 26:93–99.
19. Cover, T.L., M.K. Tummuru, P. Cao, S.A. Thompson, and M.J. Blaser. 1994. Divergence of genetic sequences for the vacuolating cytotoxin among *Helicobacter pylori* strains. *J. Biol. Chem.* 269:10566–10573.
20. Atherton, J.C., P. Cao, R.M.J. Peek, M.K. Tummuru, M.J. Blaser, and T.L. Cover. 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vacA* types with cytotoxin production and peptic ulceration. *J. Biol. Chem.* 270:17771–17777.
21. Ogura, K., F. Kanai, S. Maeda, H. Yoshida, M. Ogura, K. Lan, K. Hirota, T. Kawabe, Y. Shiratori, and M. Omata. 1997. High prevalence of cytotoxin positive *Helicobacter pylori* in patients unrelated to the presence of peptic ulcers in Japan. *Gut.* 41:463–468.
22. Fox, J.G., P. Correa, N.S. Taylor, N. Thompson, E. Fontham, F. Janney, M. Sobhan, B. Ruiz, and F. Hunter. 1992. High prevalence and persistence of cytotoxin-positive *Helicobacter pylori* strains in a population with high prevalence of atrophic gastritis. *Am. J. Gastroenterol.* 87:1554–1560.
23. Tee, W., J.R. Lambert, and B. Dwyer. 1995. Cytotoxin production by *Helicobacter pylori* from patients with upper gastrointestinal tract diseases. *J. Clin. Microbiol.* 33:1203–1205.
24. Pai, R., T.L. Cover, and A.S. Tarnawski. 1999. *Helicobacter pylori* vacuolating cytotoxin (VacA) disorganizes the cytoskeletal architecture of gastric epithelial cells. *Biochem. Biophys. Res. Commun.* 262:245–250.
25. Dubois, A., D.E. Berg, E.T. Incecik, N. Fiala, L.M. Heman-Ackah, G.I. Perez-Perez, and M.J. Blaser. 1996. Transient and persistent experimental infection of nonhuman primates with *Helicobacter pylori*: implications for human disease. *Infect. Immun.* 64:2885–2891.
26. Dubois, A., N. Fiala, L.M. Heman-Ackah, E.S. Drazek, A. Tarnawski, W.N. Fishbein, G.I. Perez-Perez, and M.J. Blaser. 1994. Natural gastric infection with *Helicobacter pylori* in monkeys: a model for spiral bacteria infection in humans. *Gastroenterology.* 106:1405–1417.
27. Fox, J.G., M. Batchelder, R. Marini, L. Yan, L. Handt, X. Li, B. Shames, A. Hayward, J. Campbell, and J.C. Murphy. 1995. *Helicobacter pylori*-induced gastritis in the domestic cat. *Infect. Immun.* 63:2674–2681.
28. Karita, M., Q. Li, D. Cantero, and K. Okita. 1994. Establishment of a small animal model for human *Helicobacter pylori* infection using germ-free mouse. *Am. J. Gastroenterol.* 89:208–213.
29. Lambert, J.R., M. Borromeo, K.J. Pinkard, H. Turner, C.B. Chapman, and M.L. Smith. 1987. Colonization of gnotobiotic piglets with *Campylobacter Pyloridis*—an animal model? *J. Infect. Dis.* 155:1344.
30. Radin, M.J., K.A. Eaton, S. Krakowka, D.R. Morgan, A. Lee, G. Otto, and J. Fox. 1990. *Helicobacter pylori* gastric infection in gnotobiotic beagle dogs. *Infect. Immun.* 58:2606–2612.
31. Shuto, R., T. Fujioka, T. Kubota, and M. Nasu. 1993. Experimental gastritis induced by *Helicobacter pylori* in Japanese monkeys. *Infect. Immun.* 61:933–939.
32. Marchetti, M., B. Arico, D. Burroni, N. Figura, R. Rappuoli, and P. Ghiara. 1995. Development of a mouse model of *Helicobacter pylori* infection that mimics human disease. *Science.* 267:1655–1658.
33. Hirayama, F., S. Takagi, H. Kusuhara, E. Iwao, Y. Yokoyama, and Y. Ikeda. 1996. Induction of gastric ulcer and intestinal metaplasia in Mongolian gerbils infected with *Helicobacter pylori*. *J. Gastroenterol.* 31:755–757.
34. Watanabe, T., M. Tada, H. Nagai, S. Sasaki, and M. Nakao.

1998. *Helicobacter pylori* infection induces gastric cancer in Mongolian gerbils. *Gastroenterology*. 115:642–648.
35. Ikeno, T., H. Ota, A. Sugiyama, K. Ishida, T. Katsuyama, R.M. Genta, and S. Kawasaki. 1999. *Helicobacter pylori*-induced chronic active gastritis, intestinal metaplasia, and gastric ulcer in Mongolian gerbils. *Am. J. Pathol.* 154:951–960.
36. Wirth, H.P., M.H. Beins, M. Yang, K.T. Tham, and M.J. Blaser. 1998. Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. *Infect. Immun.* 66:4856–4866.
37. Peek, R.M., Jr., H.P. Wirth, S.F. Moss, M. Yang, A.M. Abdalla, K.T. Tham, T. Zhang, L.H. Tang, I.M. Modlin, and M.J. Blaser. 2000. *Helicobacter pylori* alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. *Gastroenterology*. 118:48–59.
38. Maeda, S., H. Yoshida, T. Ikenoue, K. Ogura, F. Kanai, N. Kato, Y. Shiratori, and M. Omata. 1999. Structure of *cag* pathogenicity island in Japanese *Helicobacter pylori* isolates. *Gut*. 44:336–341.
39. Tomb, J.F., O. White, A.R. Kerlavage, R.A. Clayton, G.G. Sutton, R.D. Fleischmann, K.A. Ketchum, H.P. Klenk, S. Gill, B.A. Dougherty, et al. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*. 338: 539–547.
40. Phadnis, S.H., D. Ilver, L. Janzon, S. Normark, and T.U. Westblom. 1994. Pathological significance and molecular characterization of the vacuolating cytotoxin gene of *Helicobacter pylori*. *Infect. Immun.* 62:1557–1565.
41. Dale, A., J.E. Thomas, M.K. Darboe, W.A. Coward, M. Harding, and L.T. Weaver. 1998. *Helicobacter pylori* infection, gastric acid secretion, and infant growth. *J. Pediatr. Gastroenterol. Nutr.* 26:393–397.
42. Fall, C.H., P.M. Goggin, P. Hawtin, D. Fine, and S. Duggleby. 1997. Growth in infancy, infant feeding, childhood living conditions, and *Helicobacter pylori* infection at age 70. *Arch. Dis. Child.* 77:310–314.
43. Clemens, J., M.J. Albert, M. Rao, S. Huda, F. Qadri, F.P. Van Loon, B. Pradhan, A. Naficy, and A. Banik. 1996. Sociodemographic, hygienic and nutritional correlates of *Helicobacter pylori* infection of young Bangladeshi children. *Pediatr. Infect. Dis. J.* 15:1113–1118.
44. Quinonez, J.M., F. Chew, O. Torres, and R.E. Begue. 1999. Nutritional status of *Helicobacter pylori*-infected children in Guatemala as compared with uninfected peers. *Am. J. Trop. Med. Hyg.* 61:395–398.
45. Kehrt, R., M. Becker, H. Brosicke, N. Kruger, and H. Helge. 1997. Prevalence of *Helicobacter pylori* infection in Nicaraguan children with persistent diarrhea, diagnosed by the 13C-urea breath test. *J. Pediatr. Gastroenterol. Nutr.* 25: 84–88.
46. Kersulyte, D., H. Chalkauskas, and D.E. Berg. 1999. Emergence of recombinant strains of *Helicobacter pylori* during human infection. *Mol. Microbiol.* 31:31–43.
47. Akopyants, N.S., S.W. Clifton, D. Kersulyte, J.E. Crabtree, B.E. Youree, C.A. Reece, N.O. Bukanov, E.S. Drazek, B.A. Roe, and D.E. Berg. 1998. Analyses of the cag pathogenicity island of *Helicobacter pylori*. *Mol. Microbiol.* 28:37–53.
48. Glocker, E., C. Lange, A. Covacci, S. Bereswill, M. Kist, and H.L. Pahl. 1998. Proteins encoded by the *cag* pathogenicity island of *Helicobacter pylori* are required for NF-kappaB activation. *Infect. Immun.* 66:2346–2348.
49. Covacci, A., J.L. Telfold, G.D. Giudice, J. Parsonnet, and R. Rappuoli. 1999. *Helicobacter pylori* virulence and genetic geography. *Science*. 284:1328–1333.
50. Kuipers, E.J. 1999. Review article: exploring the link between *Helicobacter pylori* and gastric cancer. *Aliment. Pharmacol. Ther.* 13 (Suppl.) 1:3–11.
51. Telford, J.L., P. Ghiara, M. Dell'Orco, M. Comanducci, D. Burroni, M. Bugnoli, M.F. Tecce, S. Censini, A. Covacci, Z. Xiang, et al. 1994. Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *J. Exp. Med.* 179:1653–1658.
52. Xiang, Z., S. Censini, P.F. Bayeli, J.L. Telford, N. Figura, R. Rappuoli, and A. Covacci. 1995. Analysis of expression of CagA and VacA virulence factors in 43 strains of *Helicobacter pylori* reveals that clinical isolates can be divided into two major types and that CagA is not necessary for expression of the vacuolating cytotoxin. *Infect. Immun.* 63:94–98.
53. Maeda, S., K. Ogura, H. Yoshida, F. Kanai, T. Ikenoue, N. Kato, Y. Shiratori, and M. Omata. 1998. Major virulence factors, VacA and CagA, are commonly positive in *Helicobacter pylori* isolates in Japan. *Gut*. 42:338–343.
54. Satin, B., N. Norais, J. Telford, R. Rappuoli, M. Murgia, C. Montecucco, and E. Papini. 1997. Effect of *Helicobacter pylori* vacuolating toxin on maturation and extracellular release of procathepsin D and on epidermal growth factor degradation. *J. Biol. Chem.* 272:25022–25028.
55. Ghiara, P., M. Marchetti, M.J. Blaser, M.K. Tummuru, T.L. Cover, E.D. Segal, L.S. Tompkins, and R. Rappuoli. 1995. Role of the *Helicobacter pylori* virulence factors vacuolating cytotoxin, CagA, and urease in a mouse model of disease. *Infect. Immun.* 63:4154–4160.