

Host Cell Responses to Genotypically Similar *Helicobacter pylori* Isolates from United States and Japan

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Associations of *Helicobacter pylori* genotypes with disease differ between Western countries and Asia. Therefore, we directly compared histopathological and in vitro responses to clinical isolates with similar genotypes. Sixty-three *cagA*⁺ *vacAs1/m1* *H. pylori* isolates (United States, *n* = 24; Japan, *n* = 39) and eight *cagA*-negative *vacAs2/m2* strains were incubated with AGS cells, and supernatants were assayed for interleukin-8 (IL-8) and for DNA fragmentation. CagA tyrosine phosphorylation in AGS cells and the sequence of the putative HP0638 (*oipA*) signal sequence region were determined for 22 representative strains. HP0638 and/or *cag* island mutant strains were created and examined in IL-8 and CagA tyrosine phosphorylation assays. Levels of IL-8 induction and DNA fragmentation were similar in the U.S. and Japanese *cagA*⁺ *vacAs1/m1* isolates. All 10 of the isolates with the highest IL-8 induction and 8 of the 10 isolates with the lowest IL-8 induction had an in-frame *oipA* open reading frame, and all 10 of the isolates with the highest IL-8 induction and 7 of the 10 isolates with the lowest IL-8 induction induced CagA tyrosine phosphorylation in AGS cells. Eight isolates from gastric ulcer patients induced significantly more apoptosis in vitro, and more severe gastritis and atrophy in vivo, than other Japanese isolates. Disruption of HP0638 did not affect IL-8 induction or CagA tyrosine phosphorylation. Thus, *H. pylori* *cagA*⁺ *vacAs1/m1* isolates from the United States and Japan induce similar IL-8 and apoptosis levels. Inactivation of HP0638 does not alter epithelial responses mediated by the *cag* island in vitro. Assessment of apoptosis in vitro identified a group of *H. pylori* isolates that induce more severe gastric inflammation and atrophy.

Carriage of *Helicobacter pylori* increases the risk for developing peptic ulcer disease and gastric cancer (14). Persistent colonization results in mucosal release of chemotactic factors that attract neutrophils and mononuclear cells (21, 35, 39), and *H. pylori* induces release of the neutrophil chemoattractant chemokines from gastric epithelial cells in vitro (20, 22). In particular, interleukin-8 (IL-8) recruits and activates neutrophils (2), but mucosal IL-8 levels vary considerably among colonized individuals (5). There are several nonconserved markers of *H. pylori* virulence, including particular vacuolating cytotoxin genotypes (*vacA* genotypes); the presence of *cagA*, which is a marker for the *cag* pathogenicity island; the *iceA1* allele; and the ability to directly activate neutrophils (11, 16, 17, 25, 47, 58, 63). In vivo, *cagA*⁺ strains are associated with higher levels of mucosal IL-8 than are *cagA*-negative strains (20, 46). In vitro, IL-8 production from gastric epithelial cells following coculture with *cagA*⁺ strains is dependent upon particular *cag* island genes (up-regulating or down-regulating IL-8 synthesis) (19, 32, 59) and possibly on HP0638 (*oipA*), which encodes a putative outer membrane protein (67). Attachment of *H. pylori* to epithelial cells in vitro induces another cellular phenotype, tyrosine phosphorylation of the CagA protein, af-

ter CagA injection into cells via a type IV secretion system (42). Mutations in several genes of the *cag* pathogenicity island interfere with tyrosine phosphorylation as well as IL-8 induction (15, 32, 54). *H. pylori* colonization also affects human gastric epithelial cell apoptosis and proliferation (28, 36, 37, 38, 48). Variation in epithelial cell cycle characteristics appears to be related to particular *H. pylori* genotypes (30, 37, 50, 51, 66).

In addition to *H. pylori* allelic differences that are conserved worldwide, certain genotypes are geographically related. In particular, *vacA* and *cagA* sequence motifs in strains from the United States and Europe differ from those predominating in East Asia (1, 10, 27, 44, 60, 61, 64). In Western populations, *cagA*⁺ *vacAs1/m1* *H. pylori* strains are clearly more highly associated with disease than are *cagA*-negative *vacAs2/m2* strains (7, 63); however, since a high proportion of East Asian strains are *cagA*⁺ *vacAs1* (34), it has been difficult to detect disease-specific associations in this locale. An alternative hypothesis is that host variation is important in determining population-based differences in disease outcomes (23, 43, 52).

Therefore, we sought to determine whether there are differences in virulence between Western and East Asian *H. pylori* strains of similar genotype. To do this, we directly compared *cagA*⁺ *vacAs1/m1* *H. pylori* strains from the United States and from Japan for their ability to induce IL-8 secretion, apoptosis, and CagA phosphorylation in gastric epithelial cells in vitro. These data then were compared to histological severity of inflammation and injury in the corresponding source patients.

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TABLE 1. Characteristics of the 71 *H. pylori* isolates and their respective source patients

Country, <i>cagA</i> status, and endoscopic diagnosis	No. of patients	Age, yr (mean \pm SD)	% Male	No. with the following <i>vacA</i> (s) genotype:			
				s1a	s1b	s1c	s2
United States							
<i>cagA</i> ⁺ ^a							
DU	10	58 \pm 15	100	4	6	0	0
GU	2	53 \pm 7	100	1	1	0	0
NUD	12	52 \pm 18	100	2	10	0	0
Total	24	54 \pm 16	100	7	17	0	0
<i>cagA</i> negative ^b	8	58 \pm 11	100	0	0	0	8
Japan, <i>cagA</i> ⁺ ^a							
DU	9	45 \pm 11	78	0	0	9	0
GU	17	49 \pm 6	94	1	0	16	0
NUD	7	43 \pm 11	86	1	0	6	0
Gastric cancer	6	60 \pm 13	67	0	0	6	0
Total	39	49 \pm 11	85	2	0	37	0

^a For each of these patients, the isolate was *cagA*⁺ *vacAs1*m1.

^b Four patients had NUD, and one patient had DU; all strains were *vacAs2*m2.

MATERIALS AND METHODS

Study groups and biopsies. We studied *H. pylori* strains isolated from 24 U.S. and 39 Japanese patients undergoing gastroscopy for evaluation of upper gastrointestinal tract symptoms, as described previously (6, 9, 46). At the time of endoscopy, three biopsy specimens were obtained from adjacent areas of the gastric antrum: one was for bacterial culture of *H. pylori*, one was for urease activity (CLO test; Delta West, Bentley, Australia), and one was for routine histological examination (hematoxylin-eosin and Giemsa stains). All biopsies were obtained from an area of endoscopically intact mucosa distant from any focal lesions, such as erosions. At bacterial isolation, single colonies were collected and bacteria were identified as *H. pylori* by Gram stain morphology as well as by urease and oxidase activities. These 63 isolates were selected because their *cagA* and *vacA* status had been determined by PCR using established primers (9, 46), and confirmation of *cagA* and *vacA* genotypes was established by reverse hybridization using a line probe assay (62). As controls, from the same U.S. population (46, 47), eight *cagA*-negative strains were included; thus, a total of 71 strains were studied (Table 1). Patients were diagnosed as having duodenal ulcer (DU), gastric ulcer (GU), or nonulcer dyspepsia (NUD), based on endoscopic findings (9, 46). Six patients from Japan had been diagnosed with gastric cancer based on histological examination in addition to endoscopic findings (Table 1) (31).

Histology. Neutrophil infiltration, mononuclear cell infiltration, glandular atrophy, intestinal metaplasia, and *H. pylori* density in the gastric biopsies were assessed on a scale of 0 to 3 according to the Sydney system (49), using formalin-fixed biopsy tissues stained with hematoxylin-eosin or Giemsa. All histological evaluations were performed by a single pathologist without knowledge of the experimental results.

AGS cell culture and IL-8 assays. AGS human gastric epithelial cells (ATCC CRL 1739) were grown in RPMI 1640 (Life Technologies, Inc., Rockville, Md.) supplemented with 10% fetal bovine serum (FBS) and 20 μ g of gentamicin per ml in an atmosphere of 5% CO₂ at 37°C. For coculture experiments, *H. pylori* was grown in brucella broth with 5% FBS for 48 h, and cells were harvested by centrifugation (2,000 \times g) and resuspended in antibiotic-free RPMI 1640 with 10% FBS to yield a final concentration of 10⁸ CFU/ml. *H. pylori* was added to AGS cells at a bacteria/cell ratio of 1,000:1 for the IL-8 assays or 100:1 for the apoptosis assays (45, 55). Experiments were performed in antibiotic-free medium with 10% FBS using T-150 flasks (Corning Costar, Cambridge, Mass.) or 96-well polypropylene tissue culture plates (Nunc, Roskilde, Denmark). Levels of IL-8 (picograms per milliliter) in culture supernatants were assayed in duplicate using a specific enzyme-linked immunosorbent assay (ELISA) (Research and Development Systems, Minneapolis, Minn.) according to the manufacturer's instructions.

Assessment of apoptosis. DNA fragmentation was quantified using a commercially available ELISA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) that detects nucleosomal fragments in cytoplasmic fractions of cells undergoing apoptosis but not necrosis. For these experiments, 5 \times 10⁵ AGS cells/well in 96-well plates were incubated in duplicate with *H. pylori* cells (5 \times 10⁵ CFU/well) or with RPMI 1640-10% FBS alone for 48 h and lysed. Lysates were centrifuged,

and supernatants were used for ELISA. Absorbance measured at 405 nm was compared for AGS cells cultured with *H. pylori* or with medium alone.

Assessment of HP0638 status. According to the complete genome sequences of *H. pylori* strains 26695 and J99, dinucleotide repeats are located in the region carrying the signal sequence of HP0638 (*oipA*) (4, 57, 67). The signal sequence of HP0638, including the repeat region, was amplified by PCR using primer pair AN9260 (5'-CAAGCGCTTAACAGATAGGC-3', forward) and AN9261 (5'-AAGCGTTTTCTGCTGAAGC-3', reverse) as described previously (67). Sequence determinations for amplified DNA fragments were performed at the New York University Medical Center DNA sequencing core facility.

Tyrosine phosphorylation. AGS cells (5 \times 10⁵) were cocultured with *H. pylori* at a multiplicity of infection of 100 for 4 h. Nonadherent bacteria were removed by washing five times with phosphate-buffered saline (PBS). Cells were suspended in 1 ml of ice-cold cell scraper solution (PBS, 1 mM EDTA, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 μ M leupeptin, 1 μ M pepstatin), collected by centrifugation, and resuspended in 30 μ l of PBS. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, an equal volume of 2 \times sample buffer (125 mM Tris [pH 6.8], 2% β -mercaptoethanol, 4% sodium dodecyl sulfate, 10% glycerol, 0.006% bromophenol blue) was added to the sample before boiling (5 min). Proteins were separated on a 7% polyacrylamide gel, electrotransferred to a polyvinylidene difluoride membrane, and examined for the presence of CagA using polyclonal rabbit anti-CagA immunoglobulin G (18) or phosphotyrosine (PY20; Transduction Laboratories). The horseradish peroxidase-conjugated antibody was visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech Corp., Piscataway, N.J.) as indicated by the manufacturer.

Disruption of HP0638 in *H. pylori* strains. The HP0638 locus of strain 26695 was amplified using primers HP0637F (5'-AAGCGTAAGGGAAG-3') and HP0638R (5'-ACTTTAAACGCTGGC-3'), and the PCR product was cloned into pGEMT and used to transform *Escherichia coli* DH5 α . A unique *EcoRI* site was created by inverse PCR with primers HP0638MR (5'-CGGAATCCGAGCTTTTTCATGGT-3') and HP0638MF (5'-CGGAATCCGTTCTGGCTCCACGCTG-3'). Plasmid pUC4K was digested with *EcoRI*, after which the Kan^r cassette (*aphA*) was ligated into the inverse PCR product to disrupt the HP0638 open reading frame (ORF), creating pAN0638. *H. pylori* strains 98-30, 98-11, and 98-40, which were *cagA*⁺ and had HP0638 in frame, were transformed to kanamycin resistance with pAN0638 to create 98-30-638::*aphA*, 98-11-638::*aphA*, and 98-40-638::*aphA*, respectively. Chromosomal DNA was isolated from these strains, and the insertion of the *aphA* cassette within HP0638 in the transformants was confirmed by PCR using primers AN9260 and AN9261, as described above (see Fig. 3A).

***cag* island deletion.** *cag* island deletion mutants were generated in strains 98-30, 98-11, and 98-40 and in the HP0638::*aphA* mutants of these strains by allelic exchange, using the *cat* cassette (45). Isogenic *cag* deletion mutants were selected with chloramphenicol (10 μ g/ml). Chromosomal DNA was isolated from mutant strains, and *cag* island allelic exchange with replacement by *cat* was confirmed by PCR using primers OP1690 (5'-CCAATTTCACTCGCTATGACGGCATG-3') and OP1691 (5'-AAGCTTTGTCTATTCTAAAATGCAAC-3'). The *picB* (*cag23*) locus also was amplified by PCR using primers C3814 (5'-TTGAAAACCTCAAGGATAGGATAGAGC-3') and C3815 (5'-GCCTAGCGTAAATACACCATTACCC-3') to confirm the presence or absence of the *cag* island.

Statistical analysis. Statistical analysis was performed by the χ^2 , Fisher's exact, paired *t*, or Mann-Whitney U test, depending on the data characteristics. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Assessment of *H. pylori cagA* and *vacA* status. PCR and line probe assay were performed on *H. pylori* strains isolated from symptomatic U.S. and Japanese patients to determine the genotype (Table 1). We confirmed that 63 strains (United States, *n* = 24; Japan, *n* = 39) were *cagA*⁺ *vacAs1*m1. Most of the Japanese strains were *vacA* subtype s1c, while the U.S. strains were *vacAs1a* or *vacAs1b*. Thus, although not identical, the U.S. and Japanese strains had the same *cagA* and *vacAs1*m1 status. We also confirmed that the eight control U.S. strains were *cagA* negative and *vacAs2*m2.

Histological findings. Comparison of the intensities of antral histological findings (infiltration with mononuclear or poly-

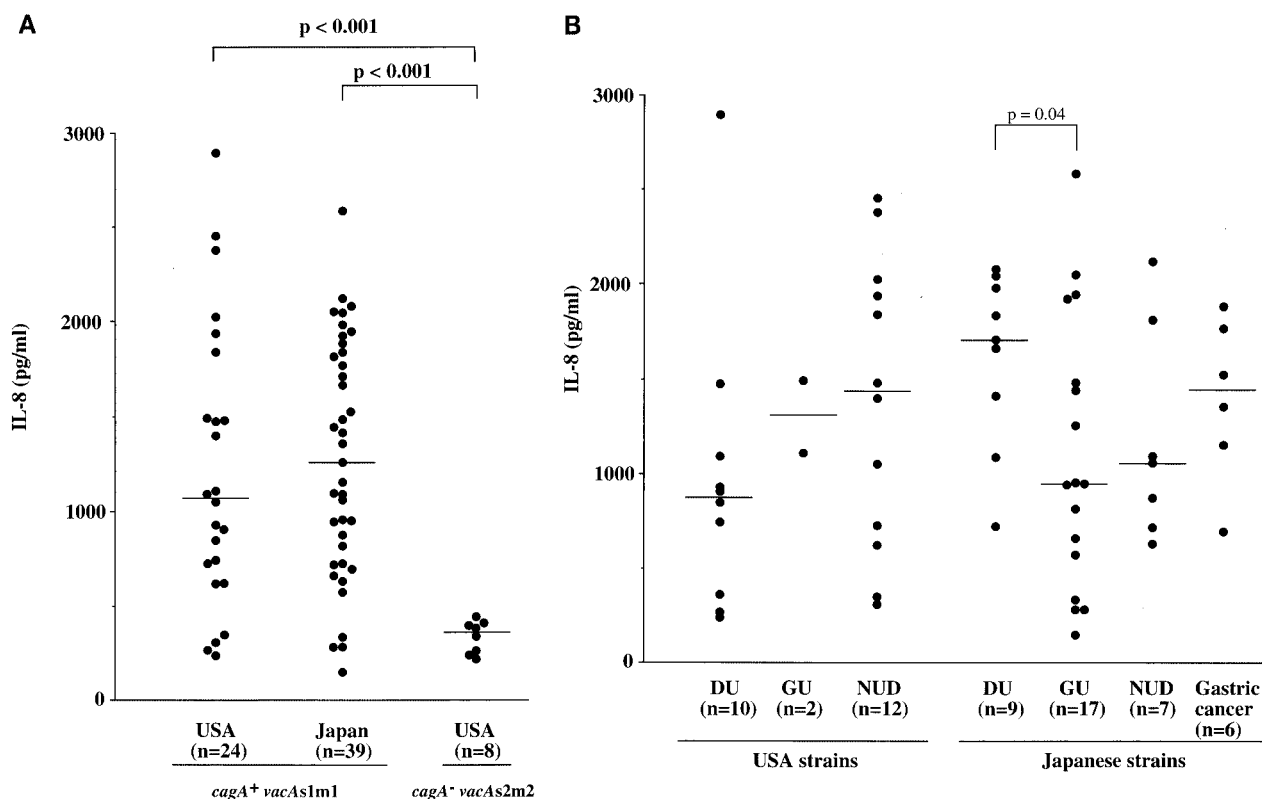


FIG. 1. IL-8 levels in culture supernatants from AGS cells coincubated with *H. pylori* cells. (A) Sixty-three *cagA*⁺ *vacAs1/m1* and eight control *cagA*-negative *vacAs2/m2* isolates were studied. IL-8 secretion by AGS cells was not significantly different between U.S. ($n = 24$) and Japanese ($n = 39$) *cagA*⁺ *vacAs1/m1* isolates, but both were significantly ($P < 0.001$) greater than that for the *cagA*-negative *vacAs2/m2* isolates. Horizontal lines indicate median values. (B) IL-8 levels according to disease outcome. Among the Japanese strains, those isolated from patients with DU induced the highest IL-8 values, but this trend was not present among U.S. strains. Horizontal lines indicate median values.

morphonuclear cells, atrophy, metaplasia, and *H. pylori* density) among the U.S. and Japanese *H. pylori*-positive patients indicated that for all five histological features, a greater proportion of persons harboring *cagA*⁺ strains showed higher scores than did *cagA*-negative patients (data not shown). No significant differences were observed in any of the histological scores between the U.S. and Japanese patients colonized with *cagA*⁺ *vacAs1/m1* isolates.

IL-8 induction in AGS cells. Gastric mucosal IL-8 levels are significantly correlated with extent of inflammatory cellular infiltration in gastric tissues of *H. pylori*-positive persons (5). To assess strain-specific cytokine induction, each of the 68 *H. pylori* isolates was incubated with AGS cells and 24-h supernatants were assayed for IL-8 release. There were no significant differences in IL-8 secretion between the U.S. (median, 1,073 pg/ml; range, 237 to 2,898 pg/ml) and the Japanese (median, 1,259 pg/ml; range, 151 to 2,590 pg/ml) *cagA*⁺ *vacAs1/m1* isolates (Fig. 1A), but both groups induced significantly ($P < 0.001$) higher levels than the *cagA*-negative *vacAs2/m2* isolates (median, 302 pg/ml; range, 203 to 472 pg/ml). We next stratified the isolates on the basis of clinical outcome. Among U.S. strains, IL-8 induction did not vary significantly according to clinical group (Fig. 1B). Among Japanese strains, those isolated from DU patients (median, 1,711 pg/ml; range, 726 to 2,081 pg/ml) induced significantly ($P = 0.04$) higher IL-8 levels than those induced by GU strains (median, 954 pg/ml; range,

151 to 2,590 pg/ml), but their ranges overlapped substantially (Fig. 1B).

AGS cell DNA fragmentation. AGS cell DNA fragmentation was no different for U.S. (median, 1.2-fold increase in DNA fragmentation relative to control; range, 0.3 to 3.4) and Japanese (median, 1.3; range, 0.5 to 5.8) *cagA*⁺ *vacAs1/m1* isolates. DNA fragmentation was significantly higher following coculture with the 17 isolates from Japanese patients with GU (median, 2.2; range, 0.7 to 5.8) than with isolates from Japanese patients with DU (median, 1.0; range, 0.5 to 2.8), NUD (median, 1.3; range, 0.6 to 1.9), or gastric cancer (median, 0.9; range, 0.6 to 1.7) ($P < 0.01$ for each) (Fig. 2A). Among the 17 Japanese GU strains, 8 induced more than a fourfold increase in DNA fragmentation relative to controls, values higher than in any group of patients (Fig. 2A, box). The demographic characteristics and *vacA* and *iceA* genotypes of these eight strains were no different from those of the isolates from the other 31 Japanese patients (Table 2), nor was IL-8 induction (median, 881 pg/ml; range, 151 to 2,590 pg/ml) significantly different from that of the other 31 Japanese isolates (median, 1,416 pg/ml; range, 335 to 2,123 pg/ml). The locations of GU in the Japanese patients harboring the eight high-apoptosis-inducing strains (body, 5; antrum, 2; fundus, 1) were similar to those in the nine Japanese patients colonized with the other strains (body, 5; antrum, 2; fundus, 2). However, histological examination of gastric biopsies from the eight GU patients

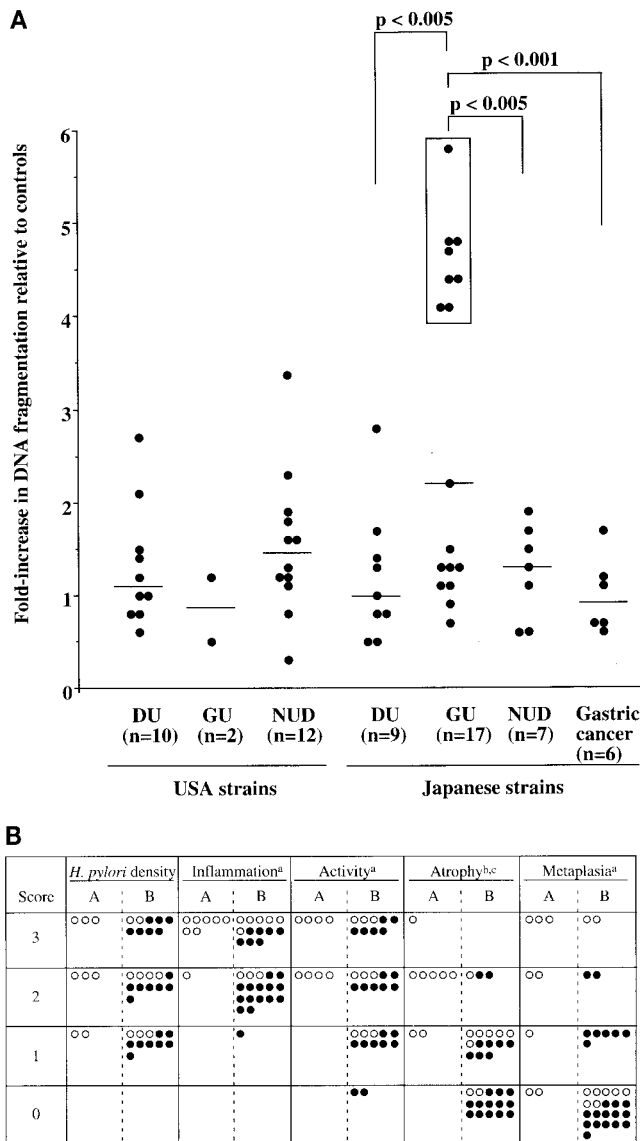


FIG. 2. (A) Apoptosis of AGS cells following coculture with *H. pylori* cells, according to clinical diagnosis of patients from whom strains were isolated. Induction of DNA fragmentation was not significantly different between U.S. ($n = 24$) and Japanese ($n = 39$) *cagA*⁺ *vacAs1/m1* isolates. Fragmentation was highest following coculture with Japanese isolates from GU patients ($P < 0.005$ for all comparisons with other isolates from Japanese patients). Horizontal lines indicate median values. For further analyses, the values for the eight isolates from GU patients that induced the highest apoptosis scores are boxed. (B) Comparison of histological findings present in the eight Japanese patients with GU from whom the isolates induced the highest apoptosis scores (columns A) and the other 31 Japanese patients (columns B), including nine other GU patients. ○, strain isolated from a patient with GU; ●, strain isolated from a patient without GU. a and b, comparison of group A patients and group B patients (a, $P < 0.05$; b, $P < 0.001$). c, comparison of group A patients and group B GU patients ($P < 0.01$).

from whom these strains were isolated showed significantly ($P < 0.05$) more severe mononuclear cell and neutrophil infiltration, atrophy, and intestinal metaplasia than in the other 31 Japanese patients (Fig. 2B). Comparison of these eight isolates

to the nine other isolates from Japanese GU patients revealed no differences in IL-8 induction or patient demographic characteristics. However, the eight GU patients with high-apoptosis-inducing *H. pylori* isolates had significantly ($P < 0.05$) more atrophy on gastric biopsy than the other nine GU patients (Fig. 2B).

Relationship between IL-8 induction, HP0638 frame status, and CagA phosphorylation. Yamaoka et al. reported that HP0638 (*oipA*) reading frame status affects the ability of *H. pylori* strains to induce IL-8 secretion (67). To further evaluate this hypothesis, we sequenced the 439-bp region that includes the putative HP0638 signal sequence for the five *cagA*⁺ *vacAs1/m1* strains with the highest and the five *cagA*⁺ *vacAs1/m1* strains with the lowest IL-8 induction from the United States and Japan and for two *cagA*-negative control strains. All 10 Japanese and 8 of the 10 U.S. strains possessed an in-frame ORF, whereas 2 of the 5 strains from the United States that least induced IL-8 had an out-of-frame HP0638 ORF (Table 3). The HP0638 ORF was out of frame in both *cagA*-negative control strains. For all 17 Japanese GU isolates, the HP0638 ORF was in frame (data not shown). We also examined the ability of the same strains to induce CagA phosphorylation in AGS cells. All 10 high-IL-8 inducing strains strongly induced tyrosine-phosphorylated CagA in AGS cells, compared with 7 of 10 low-IL-8 inducing strains (Table 3). There was no relationship to HP0638 frame status. As expected, neither *cagA*-negative control strain induced CagA tyrosine phosphorylation in AGS cells. Seven of the eight GU isolates with the highest apoptosis induction induced CagA phosphoprotein in AGS cells (Table 2).

AGS cell IL-8 induction and CagA tyrosine phosphorylation by mutant strains. To directly assess the role of HP0638 and the *cag* island in IL-8 induction, we generated HP0638 mutants, *cag* mutants, and HP0638 and *cag* mutants of strains 98-30, 98-11, and 98-40, all of which were strong IL-8 inducers (Fig. 3A). Each of the HP0638 mutant strains induced an IL-8 level similar to that of its wild-type strain. In contrast, each *cag* mutant strain induced significantly lower IL-8 levels than its wild-type or HP0638 mutant strain (Fig. 3B). There was no difference in IL-8 induction between *cag* mutant strains and HP0638 and *cag* mutant strains. Next, we examined the effects of loss of the *cag* island and/or HP0638 status on CagA tyrosine phosphorylation using wild-type and mutant strains of 98-30, 98-11, and 98-40. All wild-type and HP0638 mutant strains strongly induced tyrosine phosphorylation of the CagA protein in AGS cells, whereas the *cag* mutant and HP0638 and *cag* mutant strains did not stimulate CagA tyrosine phosphorylation (Table 4; Fig. 3C).

DISCUSSION

Among *H. pylori*-positive patients, serological detection of infection with *cagA*⁺ strains is at present the best clinically available assay for virulence differences (8). Although *cagA*⁺ *vacAs1/m1* *H. pylori* strains are common in both East Asian and Western populations (10, 61, 64) and these strains are associated with atrophic gastritis and gastric cancer (7, 63), the difference in prevalence of gastric cancers between the two locales is not proportional to the differential prevalence of these strains. Since virulence assays have been done with strains

TABLE 2. Characteristics of eight *H. pylori* isolates with high in vitro apoptosis scores from Japanese patients with GU

Strain	Patient age (yr)/sex ^a	Apoptosis score ^b	Genotype			HP0638 frame status	IL-8 induction (pg/ml) ^c	P-Tyr ^d
			<i>cagA</i>	<i>vacAs</i>	<i>vacAm</i>			
98-38	51/M	5.8	+	slc	ml	In	817	+
98-35	42/M	4.8	+	slc	ml	In	1,929	+
98-41	46/M	4.8	+	slc	ml	In	944	+
98-39	48/M	4.7	+	slc	ml	In	954	+
98-36	46/M	4.4	+	slc	ml	In	287	+
98-37	50/F	4.4	+	slc	ml	In	287	+
98-32	36/M	4.1	+	slc	ml	In	151	-
98-40	65/M	4.1	+	slc	ml	In	2,590	+

^a M, male; F, female.

^b Fold increase in DNA fragmentation of AGS cells relative to control cells.

^c Means from two replicate assays.

^d Presence of tyrosine-phosphorylated CagA protein within epithelial cells, determined as described in Materials and Methods (+, present; -, absent).

from either the United States or Japan, our study sought to examine comparable strains from the two locales in parallel in the same assays done in a single laboratory. However, at least in part due to the enormous diversity of *H. pylori* strains (56), while we studied similar organisms (all were *cagA*⁺ *vacAs1*/m1), the U.S. and Japanese groups were not identical. For example, most Japanese isolates were slc, whereas most from the United States were slb. However, *cagA* status and *vacAs1* status appear to be the most important known determinants of

differences in inflammatory responses to *H. pylori* (12, 40, 65). The substantial overlap in the range of histological responses and IL-8 induction between the U.S. and Japanese isolates and the large differences from the U.S. *cagA*-negative *vacAs2*/m2 strains confirm the centrality of that distinction. Yamaoka et al. also reported that when compared among Japanese strains, the levels of IL-8 mRNA expression in gastric mucosa were significantly higher in *cagA*-positive than in *cagA*-negative specimens (68).

Nevertheless, IL-8 levels were variable among the *cagA*⁺ *vacAs1*/m1 isolates, regardless of origin. IL-8 induction by the Japanese DU strains is higher than that by Japanese GU strains or U.S. DU strains. Although our results do not suggest that differences in HP0638 underlie these findings, it is possible that differences in gene expression within the *cag* island and/or differences in *cag* genetic composition may contribute to these findings. Techniques such as DNA microarray hybridization have recently been utilized to more comprehensively characterize genetic content between *H. pylori* isolates, and differences in *cag* island content are directly related to induction of epithelial cell responses such as IL-8 production (26, 53). Application of such techniques to these strains is currently under way in our laboratory. Yamaoka et al. (67) suggested that the HP0638 frame status affects the ability of *H. pylori* strains to induce gastric epithelial cell IL-8 secretion. When we tested representative strains for HP0638 frame status, most (90%) were in frame, regardless of IL-8 induction (Table 3). In addition, experimental disruption of HP0638 had no effect on IL-8 induction or CagA tyrosine phosphorylation in AGS cells. Thus, in the strain population we studied, HP0638 status does not directly affect IL-8 induction, in contrast to the report by Yamaoka et al. (67). This may be due to the fact that we studied different strains. Whether or not HP0638 affects IL-8 induction cannot be assessed solely from these two studies, and therefore, further independent studies will be required to examine this relationship. While the *cag* island is largely inherited as a single 27-gene block, strains possessing partial *cag* islands have been identified (3, 13, 33, 41, 53). Recently, we reported that a *cagA*⁺ *vacAs1*/m1 strain with a large deletion of the *cag* island induced lower IL-8 levels than a *vacAs1*/m1 strain with an intact *cag* island and that partial or complete *cag* island disruption attenuated IL-8 induction in vitro and significantly decreased gastric inflammation in vivo (26). The fact

TABLE 3. HP0638 frame status of *H. pylori* isolates that induce either high or low levels of IL-8

Country and <i>H. pylori</i> isolates	Strain	IL-8 induction ^a (pg/ml)	HP0638 frame status	P-Tyr ^b	
United States <i>cagA</i> ⁺ <i>vacAs1</i> m1 isolates					
High IL-8 inducers	97-141	1,936	In	+	
	97-31	2,023	In	+	
	97-511	2,381	In	+	
	97-112	2,452	In	+	
	95-58	2,898	In	+	
	98-238	265	In	+	
Low IL-8 inducers	95-79	305	Out	-	
	96-32	350	In	-	
	96-62	362	In	+	
	96-71	620	Out	+	
<i>cagA</i> negative <i>vacAs2</i> m2 isolates					
	J188	290	Out	-	
	J292	366	Out	-	
Japan, <i>cagA</i> ⁺ <i>vacAs1</i> m1 isolates					
High IL-8 inducers	98-15	2,051	In	+	
	98-30	2,053	In	+	
	98-14	2,081	In	+	
	98-46	2,123	In	+	
	98-40	2,590	In	+	
	98-32	151	In	-	
Low IL-8 inducers	98-36	287	In	+	
	98-37	287	In	+	
	98-33	335	In	+	
	98-28	574	In	+	

^a IL-8 levels in supernatants from AGS cells cocultured with specified *H. pylori* isolates; means from duplicate determinations.

^b Presence of tyrosine-phosphorylated CagA protein within epithelial cells, determined as described in Materials and Methods (+, present; -, absent).

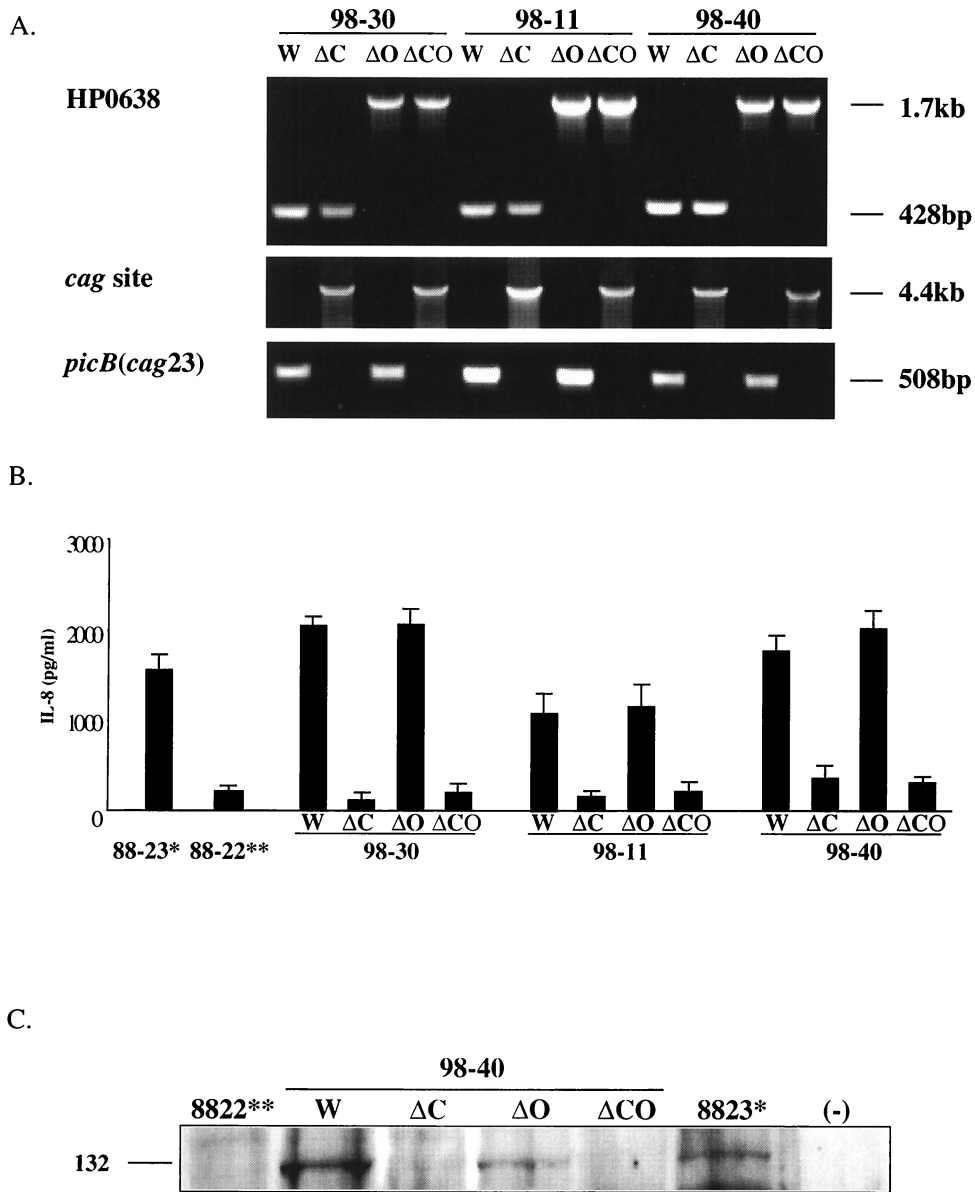


FIG. 3. Effect of mutagenizing HP0638 and the *cag* island on AGS cell responses. (A) Confirmation of mutations interrupting HP0638 and/or deleting the *cag* island. PCR was performed with primers AN9260 and AN9261 (HP0638), OP1690 and OP1691 (*cag* site), and C3814 and C3815 (*picB*) to ascertain mutant construction. W, wild type; ΔC, *cag* island deleted; ΔO, HP0638 interrupted; ΔCO, HP0638 interrupted and *cag* island deleted. (B) IL-8 levels in culture supernatants from AGS cells coincubated with *H. pylori* wild-type and mutant strains. Data represent the means and standard deviations from three experiments. *, *cagA*⁺ control strain 88-23, **, *cagA*-negative control strain 88-22. (C) Induction of tyrosine-phosphorylated CagA protein by *H. pylori* wild-type and mutant strains in AGS cells.

that certain *cagA*⁺ *vacAs1/m1* strains induce only low levels of IL-8 (Table 3) and do not induce CagA tyrosine phosphorylation in AGS cells suggests the presence of partial *cag* island deletions, which might also explain the previous findings of Yamaoka et al. (67).

Although the ability of *cagA*⁺ *vacAs1/m1* *H. pylori* strains to induce AGS cell apoptosis was not significantly different between U.S. and Japanese isolates, this study identified eight isolates that induced very high levels of apoptosis. These high-apoptosis-inducing isolates were associated with more severe mononuclear cell and neutrophil tissue infiltration, atrophy,

and intestinal metaplasia. However, we used only one biopsy for histological analysis, and since *H. pylori* colonization may be patchy, sampling errors may affect the results. Therefore, further studies should be done using multiple samples to confirm the correlation between apoptosis and histological features of the gastric mucosa. The fact that all eight strains were from Japanese patients with GU suggests that they may represent a unique group of isolates with enhanced pathogenic potential. Comparison of these isolates to those from other GU patients showed no differences in *cagA*, *vacA*, or HP0638 status or in IL-8 induction levels. Taken together, these eight

TABLE 4. Induction of CagA tyrosine phosphorylation by wild-type and mutant *H. pylori* strains

<i>H. pylori</i> strain	Presence of phosphotyrosine-CagA ^a for:			
	Wild type	<i>cag</i> island deleted	HP0638 disrupted	<i>cag</i> island deleted and HP0638 disrupted
98-30	+	–	+	–
98-11	+	–	+	–
98-40	+	–	+	–

^a Presence of tyrosine-phosphorylated CagA protein within AGS epithelial cells, determined as described in Materials and Methods (+, present; –, absent).

isolates appear to have a new phenotype: high induction of apoptosis in vitro coupled with enhanced atrophy in vivo in their human hosts. Although the genetic bases of this phenomenon are not known, these strains may represent a group of *H. pylori* strains with enhanced virulence. Testing this hypothesis in suitable animal models will be of great interest. Although we hypothesize that apoptosis is related to development of gastric cancer, the isolates from gastric cancer patients did not induce high levels of apoptosis in vitro. One hypothesis to explain these events is that colonizing strains change in their ability to induce epithelial cell responses, such as apoptosis, as an adaptation to changing conditions within the host milieu. Alternatively, our findings may not represent the true relationship between induction of apoptosis in vitro and gastric cancers, as we examined only six strains isolated from patients with gastric adenocarcinoma. To more completely address this question, apoptosis studies should be done using *H. pylori* isolates obtained from more gastric cancer patients at different stages of disease.

Apoptosis induction by the U.S. strains in the present study was lower than we have previously reported (45). There are several possibilities for this apparent discordance. First, the U.S. *H. pylori* strains used in this study differ from those in the previous study, and differences in expression of known virulence determinants (i.e., *vacA* and *picB*) and/or unidentified genetic differences may have contributed to the variation in apoptosis induction. Second, in the present study, we used a DNA fragmentation ELISA, while in the previous study, flow cytometry was used not only to assess apoptosis but also to determine the cell cycle distribution following coculture with clinical strains. Finally, apoptosis in the present study was assessed after 48 h of incubation, while the 72-h time point was chiefly used in our previous study. Collectively, variation in the strain population and these methodologic differences could have led to the differences in apoptosis levels seen in the two studies.

Gastrointestinal illnesses associated with *H. pylori* also may be related to inappropriately regulated gastric immune responses (24). Clearly, host differences between persons in the United States and Japan may play a role in the different clinical outcomes in *H. pylori*-positive persons. For example, particular polymorphisms of the human IL-1 β gene promoter are risk factors for both atrophic gastritis and gastric adenocarcinoma among *H. pylori*-positive persons (23). Host differences in other cytokine (IL-8 and GRO α) responses to *H. pylori* also may be important (43), as well as the age at which *H. pylori* is acquired (29).

In conclusion, *H. pylori cagA*⁺ *vacA*s1/m1 strains from the United States and Japan induce similar ranges of IL-8 secretion and DNA fragmentation in vitro. In contrast to a previous report (67), we show that HP0638 frame status does not affect epithelial cell responses related to pathogenesis. The identification of strains from GU patients that induce unusually high levels of apoptosis in vitro and that are associated with enhanced atrophy in vivo suggests a novel virulence-related phenotype that could be pursued in future studies.

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