[CANCER RESEARCH 62, 2385–2389, April 15, 2002]

A *Helicobacter pylori* Restriction Endonuclease-replacing Gene, *hrgA*, Is Associated with Gastric Cancer in Asian Strains[1]

Takafumi Ando,[2] Trudy M. Wassenaar, Richard M. Peek, Jr., Rahul A. Aras, Ariane I. Tschumi, Leen-Jan van Doorn, Kazuo Kusugami, and Martin J. Blaser

Departments of Medicine and Microbiology, New York University School of Medicine, New York, New York 10016 [T. A., R. A. A., A. I. T., M. J. B.]; First Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466-8550, Japan [T. A., K. K.]; Molecular Microbiology and Genomics Consultants, D-55576 Zotzenheim, Germany [T. M. W.]; Division of Gastroenterology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 [R. M. P.]; Delft Diagnostic Laboratory, 2625 AD Delft, the Netherlands [L-J. v. D.]; and Department of Veterans Affairs Medical Center, New York, New York 10016 [M. J. B.]

ABSTRACT

The sensitivity of *Helicobacter pylori* chromosomal DNA to *Mbo*I digestion was investigated in 208 strains from several continents. Only 11 (5%) of strains were sensitive to *Mbo*I, and it was hypothesized that *Hpy*III, a type II restriction/modification enzyme with sequence homology to *Mbo*I, mediated the protection. This was confirmed by PCR analysis of the gene locus of *hpy*III, normally composed of *hpy*IIIR and *hpy*IIIM. In all but one strain sensitive to *Mbo*I, no PCR product of *hpy*IIIR was obtained. In contrast, all strains yielded a product for *hpy*IIIM, independent of *Mbo*I phenotype. Further examination of the *hpy*III locus in strains lacking a *hpy*IIIR PCR product identified a novel gene, *hrgA*, upstream of *hpy*IIIM. All 208 strains examined had either *hpy*IIIR or *hrgA*, but not both, upstream of *hpy*IIIM. Although *hrgA* has homology with a *Campylobacter jejuni* gene (*Cj1602*), its function is not known. In Western countries, *hrgA* was more prevalent (53%) than in Asia (25%; $P < 0.0001$, χ^2). In Asia, *hrgA* was more prevalent among gastric cancer patients (18 of 43; 42%) than among noncancer patients (16 of 95; 17%; $P = 0.001$, χ^2). All 143 Asian strains tested were *cagA*$^+$, but among Western strains, *hrgA* was more prevalent in *cagA*$^+$ strains (26 of 42; 62%) than in *cagA*$^-$ strains (9 of 23; 39%; $P = 0.04$, χ^2). In coculture with epithelial cells, *hpy*IIIR and *hrgA* strains did not show any significant differences in interleukin-8 induction and apoptosis. Although a direct function for *hrgA* in virulence could not be demonstrated, our data indicate that *hrgA* is a strain-specific gene that might be associated with gastric cancer among *H. pylori* isolates from Asian patients.

INTRODUCTION

Helicobacter pylori are Gram-negative bacteria that colonize the human stomach and whose presence affects the risk of upper gastrointestinal tract diseases, including gastric cancer (1). Several strain-specific factors have been identified that potentially are markers for the differential risk associated with *H. pylori* colonization; at present, *cagA*, a marker of the *cag* pathogenicity island, has the strongest predictive value (2–6). However, in East Asia, most strains are *cagA*$^+$ regardless of clinical outcome (7–9). Thus, identification of bacterial factors involved in or serving as markers for the progression to ulceration or to gastric cancer remains desirable. During our study of R-M[3] systems in *H. pylori*, we unexpectedly discovered such a potential marker that among Asian patients with *cagA*$^+$ strains identifies those associated with gastric cancer.

H. pylori strains are highly heterogeneous in the number and nature of the type II R-M systems they carry (10–15). Type II R-M systems comprise two enzymes encoded by paired genes, a restriction endonuclease that cleaves DNA within a specific 4–8-bp sequence and a methyltransferase that specifically methylates the DNA at adenine or cytosine residues within the same sequence and thus protects the sequence from cleavage (16–18). *H. pylori* DNA is highly methylated at both adenine and cytosine residues (10), consistent with genomic sequence analyses that predicted 14 and 15 potential R-M systems for *H. pylori* strains 26695 and J99, respectively (11, 12). The *hpy*III R-M gene locus (13–15) is homologous to the *Mbo*I R-M system of *Moraxella bovis* (19), which recognizes the DNA sequence GATC, and the same recognition sequence has been confirmed for *hpy*III (13, 14).

In a previous study (20), we showed that all *H. pylori* strains examined were resistant to *Nla*III and that most (95%) were also resistant to *Mbo*I. *Nla*III is homologous to *hpy*IR, which has been called *iceA1*, and *Mbo*I is homologous to *hpy*IIIR. In some *H. pylori* strains, *iceA2* replaces *iceA1*, and strains with *iceA1* have been found to be more highly associated with peptic ulcer disease (21, 22) and gastric cancer (23) than those with *iceA2*. These associations prompted us to study the *hpy*III R-M system in more detail to determine whether there was a similar correlation with pathogenic outcomes. We assayed a collection of strains for susceptibility to *Mbo*I. When a small minority of strains was found to be sensitive to this restriction enzyme, they were examined to detect polymorphisms in their *hpy*III locus. These analyses identified a new gene, *hrgA*, that had replaced *hpy*IIIR in most *Mbo*I-sensitive strains. For most strains investigated, the patient's clinical outcome was known, allowing investigation of correlations with *hrgA/hpy*IIIR status. Our findings suggest that *hrgA* can potentially be used as a marker for virulence in the presence of *cagA*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. A total of 208 clinical isolates from different parts of the world (Table 1) were from patients with duodenal ulcers ($n = 55$), gastric ulcers ($n = 42$), gastric cancer ($n = 43$), and nonulcer dyspepsia ($n = 62$). The three Colombian strains were isolated from Hispanic persons and categorized as Western strains. For six patients (five from Thailand, one from the United States), clinical data were not available. The strains were obtained from 208 unique patients. At the time of endoscopy, two biopsy specimens were obtained from the greater curvature of the antrum, between 2 and 5 cm from the pylorus. To isolate *H. pylori*, biopsies were homogenized in 250 μl of saline, and 50 μl were plated onto trypticase soy agar with 5% sheep blood (BBL) and incubated for up to 9 days under microaerobic conditions. Single colonies were collected, and bacteria were identified as *H. pylori* by Gram's stain morphology as well as by urease and oxidase activity. All isolates were characterized by their *cagA* status (positive, $n = 185$; negative, $n = 23$). Strain JP26, from which *hrgA* was originally isolated and sequenced, is a *H. pylori* strain obtained from a gastric cancer patient in Japan.

DNA and Protein Techniques. Standard molecular techniques were used (24). *H. pylori* chromosomal DNA was prepared from cells of each strain after 48 h of growth on two agar plates as described (25). PCR reactions were performed in reaction volumes of 50 μl containing 0.5 units of Taq polymerase (Qiagen), 1.5 mM $MgCl_2$, and 200 ng of each primer. The PCR protocol (30

Received 11/19/01; accepted 2/14/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants R01DK53707, R01GM63270, R29CA77955, and R0158587 from the NIH and by the Medical Research Service of the Department of Veterans Affairs.

[2] To whom requests for reprints should be addressed, at First Department of Internal Medicine, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. Phone: 81-52-744-2144; Fax: 81-52-744-2157; E-mail: takafumia-gi@umin.ac.jp.

[3] The abbreviations used are: R-M, restriction-modification; IL, interleukin; FBS, fetal bovine serum.

Table 1 *Characteristics of the 208 H. pylori strains studied*

Origin	Clinical diagnosis[a]	*cagA* status	No. of strains
Japan ($n = 85$)	DU	+	20
	GU	+	25
	GCA	+	21
	NUD	+	19
Korea ($n = 19$)	DU	+	6
	GU	+	6
	GCA	+	7
China ($n = 28$)	DU	+	13
	GCA	+	15
Thailand ($n = 5$)	NA	+	5
India ($n = 6$)	DU	+	1
	NUD	+	5
United States ($n = 50$)	DU	+	7
		−	3
	GU	+	6
		−	1
	NUD	+	15
		−	17
	NA	+	1
Colombia ($n = 3$)	GU	+	2
		−	1
Europe ($n = 12$)	DU	+	4
		−	1
	GU	+	1
	NUD	+	6

[a] Clinical diagnosis of the patients from whom the *H. pylori* strain was isolated. DU, duodenal ulcer; GU, gastric ulcer; GCA, gastric cancer; NUD, nonulcer dyspepsia; NA, not assessed.

cycles) included a denaturing step at 94°C for 1 min, annealing at 5°C below the predicted melting temperature of the primers for 1 min, and extension at 72°C for 1 min/kb of amplification product. The primers, reflecting conserved sequences in the *hpy*III locus, were hpRf, hpRr, hpMf, hpMr, hrgAf, hrgAr, locf, and locr (Table 2 and Fig. 1).

***Mbo*I Digestion of *H. pylori* Chromosomal DNA.** Chromosomal DNA from the 208 *H. pylori* strains was subjected to digestion with *Mbo*I (New England Biolabs, Beverly, MA). For each reaction, 400 ng of DNA were incubated with 2.5 units of the enzyme and buffer supplied by the manufacturer for 2 h at 37°C. After incubation, digestion patterns were compared by electrophoresis on a 1.0% agarose gel.

***hrgA* Sequence.** For sequence analysis, the PCR product of JP26, generated with primers locf and locr, was purified using the QiaQuick PCR purification kit and the QiaQuick Gel Extraction kit. The purified PCR product was subsequently sequenced on both strands in an automated sequencer (Applied Biosystems, Inc.) in the New York University Cancer Center Core Laboratory and analyzed using Sequencer 3.1.1 (Gene Code Corp, Inc., Ann Arbor, MI). The accession no. was AF446009.

Disruption of *hrgA* in *H. pylori* strain 26695 or JP26. A chloramphenicol resistance gene (*cat*) was inserted in *hrgA* of JP26. A PCR fragment was first generated from *hrgA* of strain JP26, using primers NthrgAf and XhhrgAr (Table 2). This product was cloned into pBluescript, using *Escherichia coli* DH5α. A unique *Eco*RI site was created by inverse PCR with primers hrgAinr and hrgAinf, disrupting *hrgA*. The *cat* gene of pBSC103 (26) was amplified using primers catf and catr, which added *Eco*RI restriction sites. This cassette was ligated into the inverse PCR product, thereby disrupting *hrgA*. *H. pylori* strain JP26 was transformed to chloramphenicol resistance with the pBSC103 vector containing the *hrgA* gene interrupted by *cat*, to create JP26-*hrgA*::*cat*. Chromosomal DNA was isolated from the transformants, and the insertion of the *cat* cassette within *hrgA* was confirmed by PCR.

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% FBS and 20 μg/ml gentamicin in an atmosphere of 5% CO_2 at 37°C. For coculture experiments, *H. pylori* was grown in *Brucella* broth with 5% FBS for 48 h. Cells were harvested by

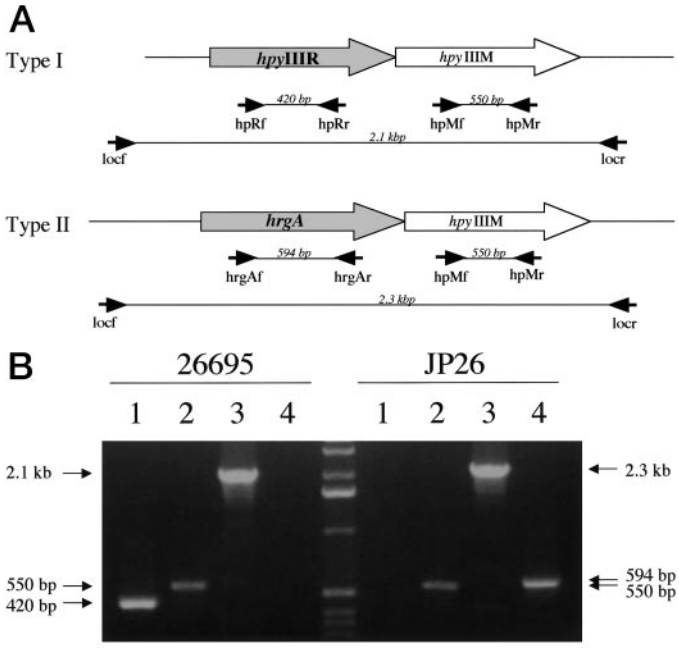

Fig. 1. *A*, schematic representation of the *hpy*III R-M locus in *H. pylori* strains. *Type I* represents strains containing *hpy*IIIR, and *Type II* represents strains containing *hrgA*. PCR primers used in the analysis of these loci are indicated by *black arrows*, and the sizes of obtained PCR products are shown in *italics*. *B*, representative agarose gel (*26695* for type I, *JP26* for type II) showing the size of PCR products obtained with the primers specific for *hpy*IIIR, *hpy*IIIM, *hrgA*, and the complete locus. For both types, *Lane 1*, *hpy*IIIR; *Lane 2*, *hpy*IIIM; *Lane 3*, the complete locus; *Lane 4*, *hrgA*.

Table 2 *Oligonucleotide primers used in this study*

Primer designation[a]	Gene locus	Location in gene[b]	Primer sequence (5′→3′)[c]
hpRf	*hpy*IIIR	226-243	CTCATTGCTGTGAGGGAT
hpRr	*hpy*IIIR	668-651	TCTTGATAGGATCTTGCG
hpMf	*hpy*IIIM	135-148	TCCGCCTTATTTCC
hpMr	*hpy*IIIM	697-684	CCGCAATGCCTGTG
hrgAf	*hrgA*	252-272	TCTCGTGAAAGAGAATTTCC
hrgAr	*hrgA*	933-914	TAAGTGTGGGTATATCAATC
locf	*HP0090*	17-1	AATAGCGCGTATTGCAT
locr	*HP0093*	422-438	CCCAAAAGTATAACGCT
NthrgAf	*hrgA*	252-272	ATAAGAATGCGGCCGCTCTCGTGAAAGAGAATTTCC
XhhrgAr	*hrgA*	933-914	CCGCTCGAGTAAGTGTGGGTATATATCAATC
hrgAinr	*hrgA*	467-448	CCGGAATTCTTTGATAAATATGACGGAG
hrgAinf	*hrgA*	572-590	CCGGAATTCTTTGATAAATATGACGGAG
catf	*cat*	−182 to −159	CCGGAATTCCGGTATCGTATGGAGCGGACAACG
catr	*cat*	674-651	CCGGAATTCGTGCGCCCTTTAGTTCCTAAAGGG
hpyIIIRf	*HP0091*	414-429	CCGGAATTCTGATAGCAATGCGAGA
hpyIIIRr	*HP0091*	413-398	CCGGAATTCAGCCCCACTTCTATAC

[a] Last letter of primer designation indicates orientation: forward (f) or reverse (r).
[b] For *hpy*IIIR, *hpy*IIIM, *HP0090*, and *HP0093*, location refers to the position within the gene in strain 26695 (11), from the first nucleotide of the initiation codon. For *hrgA*, location refers to the position within the gene in strain JP26, from the first nucleotide of the initiation codon.
[c] Added restriction sites *underlined*: GCGGCCGC (*Not*I), CTCGAG (*Xho*I), GAATTC (*Eco*RI).

Table 3 *Characterization of 208 H. pylori strains according to status of hpyIII locus and MboIR susceptibility*

Type	*Mbo*I phenotype[a]	PCR product amplified			No. of strains
		*hpy*IIIM[b]	*hpy*IIIR[c]	*hrgA*[d]	
Type IR	Resistant	+	+	−	137
Type IS	Sensitive	+	+	−	1
Type IIR	Resistant	+	−	+	60
Type IIS	Sensitive	+	−	+	10

[a] According to susceptibility to *Mbo*I digestion.
[b] PCR using primers hpMf and hpMr.
[c] PCR using primers hpRf and hpRr.
[d] PCR using primers hrgAf and hrgAr.

centrifugation (2000 × *g*) and resuspended in antibiotic-free RPMI 1640 with 10% FBS to yield a final concentration of 1×10^8 colony-forming units/ml. *H. pylori* were added to AGS cells at a bacteria:cell concentration of 1000:1 for the IL-8 assays or 100:1 for the apoptosis assays. Experiments were performed in antibiotic-free medium containing 10% FBS in T-150 flasks (Corning Costar, Cambridge, MA) or 96-well polypropylene tissue culture plates (Nunc, Roskilde, Denmark). To recapitulate events that occur in native actively replicating gastric mucosa, AGS cells were not serum starved and remained subconfluent during each assay. Levels of IL-8 (expressed as pg/ml) in culture supernatants were assayed in duplicate by specific ELISAs (Research and Development Systems, Minneapolis, MN), according to the manufacturer's instructions.

Assessment of Apoptosis by DNA Fragmentation Assay. DNA fragmentation was quantified using a commercially available ELISA (Boehringer Mannheim Biochemicals, Indianapolis, IN) that detects nucleosomal fragments in cytoplasmic fractions of cells undergoing apoptosis, but not necrosis. For these experiments, 5×10^3 AGS cells/well were incubated in 96-well plates in duplicate with *H. pylori* (5×10^5 colony-forming units/well) cells 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 between AGS cells cultured with *H. pylori* or with the negative control.

RESULTS

***Mbo*I Digestion of *H. pylori* Chromosomal DNA and PCR Analysis of the *hpy*III R-M Locus.** After a preliminary study involving a few *H. pylori* strains, chromosomal DNA from 208 strains (listed in Table 1) was digested with the restriction enzyme *Mbo*I. Of the 208 strains, 197 were resistant and 11 (5%) were sensitive. To determine whether the *Mbo*I digestion susceptibility was attributable to the absence or the genetic variability in the *hpy*III R-M gene locus (10, 13–15), the presence of *hpy*IIIR and *hpy*IIIM was assessed by PCR, using primers described in Fig. 1. With primers hpMf and hpMr, all 208 strains studied amplified a *hpy*IIIM PCR product of the expected size (550 bp). In contrast, with primers hpRf and hpRr, only 138 strains (66%) yielded a *hpy*IIIR PCR product of the expected size (420 bp). Of the 11 *Mbo*I-sensitive strains, only 1 strain (88-29) amplified a product with *hpy*IIIR-specific primers. To determine whether the absence of a *hpy*IIIR PCR product was attributable to deletion of the gene or to mutations prohibiting primer annealing, a PCR was performed using primers (locf and locr) that flank the entire *hpy*III R-M locus (Fig. 1). As expected, strains that previously showed a *hpy*IIIR PCR product now yielded the expected 2.1-kb product (except for strain 99-517, which yielded a product of ~2.0 kb). In contrast, all strains from which *hpy*IIIR had not been amplified now yielded 2.3 kb products (Fig. 1*B*). Sequence analysis of the 2.3-kb PCR product from a Japanese strain, JP26 (a *Mbo*I-sensitive strain that failed to amplify a *hpy*IIIR PCR product), revealed that another gene had replaced *hpy*IIIR. We named this gene *hrgA* (AF446009), for *H. pylori* restriction endonuclease-replacing gene A.

Prevalence of *hrgA*. The primers hrgAf and hrgAr, derived from the JP26 *hrgA* sequence, were used to examine the presence of *hrgA* in all 208 strains included in this study. The results indicated that the presence of *hrgA* and *hpy*IIIR is mutually exclusive in all strains tested (Table 3). Using PCR primer pairs hrgAf and hpMr, hrgAf and locr, and locf and hrgAr (Fig. 1), we confirmed that in all *hrgA*-positive strains, *hrgA* was located upstream of *hpy*IIIM. Strains were classified as type I when they contained *hpy*IIIR and type II when they contained *hrgA*; they were then subclassified based on the *Mbo*I susceptibility phenotype. Thus, four subtypes can be recognized (Table 3). The prevalences of these types were compared with the strain's geographic origin, *cagA* status, and clinical manifestations. Among 65 isolates from Western countries, $hrgA^+$ strains were more prevalent (52%) than among 143 Asian isolates (25%; $P < 0.001$; Table 4). Among the Western patients studied, none had gastric cancers, and $hrgA^+$ strains were more prevalent in $cagA^+$ backgrounds (26 of 42; 62%) than in $cagA^-$ backgrounds (9 of 23; 39%; $P = 0.04$). Among the 138 Asian patients studied (all $cagA^+$), $hrgA^+$ strains were more prevalent (42%) in gastric cancer patients than in patients without gastric cancer (17%; $P < 0.001$). These results indicate that *hrgA* may be a marker for strains associated with gastric cancer in Asia. There was no significant difference in *hrgA* prevalence in relation to disease outcomes (duodenal ulcer, gastric ulcer, or nonulcer dyspepsia) among nongastric cancer strains from either Asian or Western countries.

Analysis of *hrgA* Sequence. The sequence of *hrgA* was determined and a similarity search for *hrgA*, using BlastP, identified Cj1602, a hypothetical *Campylobacter jejuni* protein, as having highest homology (50% identity; 67% similarity). A weaker similarity was found with the *H. pylori* genes *HP0852* (33% identity; 50% similarity) and its orthologue *Jhp0788* (33% identity; 50% similarity). A ClustalW analysis of the putative gene product showed a conserved region lacking either α-helix or β-sheet characteristics (Fig. 2). This conserved region showed weak homology to regions in hypothetical proteins of *Xylella fastidiosa* and *Pseudomonas aeruginosa* (results not shown). The *hrgA* product is predicted to be globular, not membrane spanning, and without recognizable secretion signals, but no function was apparent from the homology or motif searches.

Role of *hrgA* in Epithelial Cell Stimulation *in Vitro*. Because *hrgA* strains have increased prevalence in isolates from Asian patients with gastric cancer, the direct role of *hrgA* in *H. pylori* virulence was examined. Because gastric mucosal IL-8 levels are significantly correlated with degree of inflammation in gastric tissue (27), we measured the induction of IL-8 release from gastric epithelial cells *in vitro* after coincubation with selected *H. pylori* strains. After coincubation of the selected *H. pylori* strains with AGS human gastric epithelial cells, supernatants were assayed for IL-8 release and for apoptosis assessment. We compared *H. pylori* strains from the United States and

Table 4 *Relationship between hpyIIIR or hrgA-containing H. pylori strains and clinical outcomes of 202 source patients[a] from whom strains were isolated*

Locality	Western strains		Asian strains		
	*hpy*IIIR ($n = 31$)	*hrgA* ($n = 33$)	*hpy*IIIR ($n = 104$)	*hrgA* ($n = 34$)	
Clinical characteristics					
Median age, yr (range)	56 (40–68)	57 (43–67)	58 (42–69)	58 (40–72)	
African-American (%)	12	14	0	0	
M:F	25:6	24:7	81:23	26:8	
Gastric cancer ($n = 43$)	0	0	25	18	
Nongastric cancer ($n = 159$)	31	33	79	16	[b]
Duodenal ulcer ($n = 55$)	6	9	34	6	
Gastric ulcer ($n = 42$)	5	6	25	6	
NUD[c] ($n = 62$)	20	18	20	4	

[a] For 6 of the original 208 strains, clinical data were not available.
[b] Among Asian strains, *hrgA* frequency in nongastric cancer cases (16 of 95; 17%) is significantly lower ($P < 0.001$) than in gastric cancer cases (18 of 43; 42%).
[c] NUD, nonulcer dyspepsia.

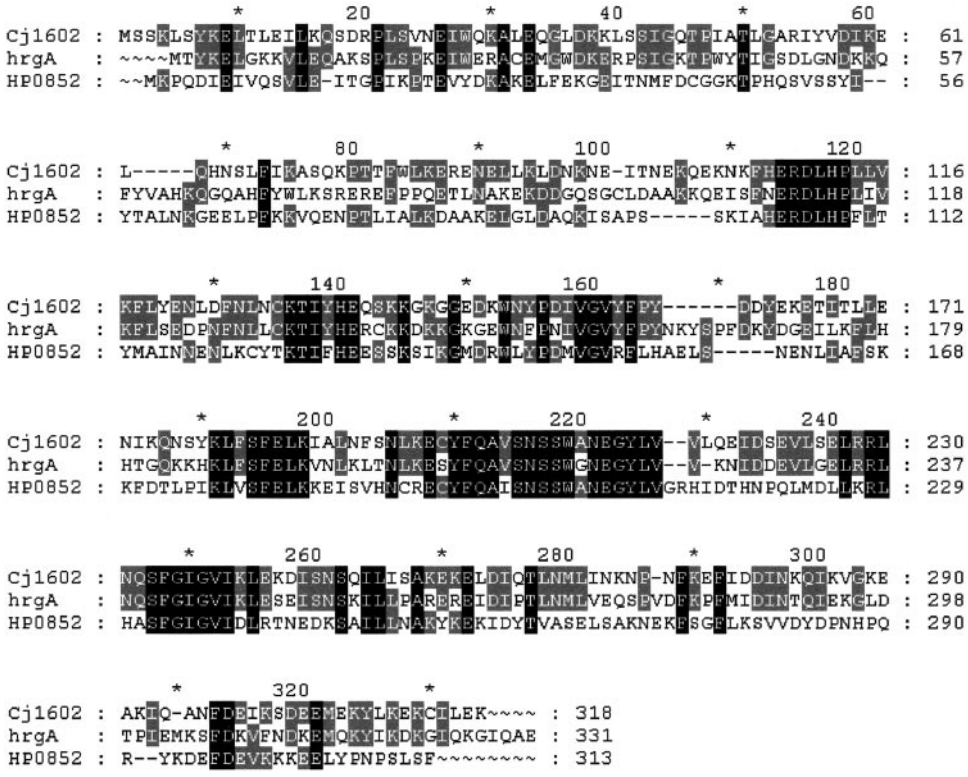

Fig. 2. ClustalW alignment of translated sequence of putative products of *Cj1602* (from *C. jejuni* strain NCTC11168), *hrgA* (from strain JP26), and *HP0852* (from strain 26695). *Black background* indicates conserved amino acid in all three strains; *gray* indicates conserved amino acid in two of the three strains.

Japan, including both *hpy*IIIR (United States, $n = 9$; Japan, $n = 33$) and *hrgA* (United States, $n = 14$; Japan, $n = 6$) genotypes. An isogenic mutant of JP26 in which *hrgA* was insertionally inactivated (JP26-*hrgA::cat*) was assessed in parallel. There was no significant difference in either induction of IL-8 or apoptosis in AGS cells when we compared the *hpy*IIIR and *hrgA* strains (Table 5), or between the strains from the United States and Japan (data not shown). The *hrgA*$^-$ mutant (JP26-*hrgA::cat*) and wild-type JP26 cells also showed essentially identical behavior in these *in vitro* models (data not shown). Thus, *hrgA* does not encode a factor responsible for the properties associated with *H. pylori* virulence that are measured in these models.

DISCUSSION

This study reports that of 208 *H. pylori* strains examined, originating from different continents, all possess *hpy*IIIM, preceded by either *hpy*IIIR or *hrgA*. No strain contains or lacks both. The organization of the *hpy*III locus parallels the *hpy*I locus, which consists of *hpy*IM and either *iceA1* (which encodes the *Hpy*IR protein) or *iceA2*, an unrelated hypothetical protein (28, 29). Activity of *Hpy*IM (CATG-specific adenine methylation) is present in 100% of the strains studied (10, 30), and as shown in this study, *Hpy*IIIM activity (GATC-specific adenine methylation) is found in 95% of *H. pylori* strains as determined by susceptibility to *Mbo*I. This frequency of methylation is substantially greater than that of other methylases among the *H. pylori* strains studied (10, 20). Of the 11 *Mbo*I-sensitive strains, only 1 strain (88-29) possessed *hpy*IIIR; additional studies on this strain, including experimental rodent infections, have been initiated. The parallels between *hpy*III and *iceA* suggest that replacement of a restriction endonuclease gene by a nonrelated gene may be common in *H. pylori*. In that sense, *iceA2* may better be renamed as *hrgB*, because it is another example of a *H. pylori* restriction endonuclease-replacing gene. The name *iceA2* is not appropriate because the gene bears no structural or functional relationship to *iceA1*, which encodes a CATG-recognizing type II restriction enzyme homologous to *Nla*III (21, 28).

Table 5 *Effect of H. pylori strains on AGS cells, according to hrgA or hpyIIIR genotype*[a]

Criterion	Mean ± SD		
	*hpy*IIIR ($n = 42$)	*hrgA* ($n = 20$)	P^b
IL-8 induction (pg/ml)	1255 ± 696	1291 ± 595	0.83
Fold increase in DNA fragmentation[c]	1.8 ± 1.4	1.5 ± 1.1	0.34

[a] *H. pylori* cells were cocultured with AGS cells at a ratio of 1000:1 and incubated for 24 h for the IL-8 induction assay, and at a ratio of 100:1 for 48 h of incubation in the DNA fragmentation assay.
[b] Based on paired *t* test.
[c] Relative to controls.

Our findings suggest that *hrgA*, a strain-specific gene, is associated with gastric cancer among *H. pylori* isolates from Asian patients. Differing recovery rates of *hrgA*$^+$ *versus hrgA*$^-$ strains attributable to differential growth may introduce bias, but because we have not observed differences in growth rates between *hrgA*$^+$ and *hrgA*$^-$ strains *in vitro* (data not shown), this seems less likely. In previous reports, the *vacA* s1 genotype and *cagA* or *iceA1* positivity have been identified as markers of strains associated with ulcer disease or gastric cancer among Western populations (21–23, 31). Such a pattern is not immediately evident in East Asia, where regardless of the clinical status of the patient, nearly all isolates are *vacA* s1 and *cagA* positive (9, 32) and *iceA1* status does not have predictive value (9). We could not determine the correlation between *hrgA* and gastric cancer among Western strains because, at present, our strain collection does not contain a sufficient number of cancer strains from Western countries for adequate comparisons to be made. However, we have initiated collaborations to obtain such strains to address this question in future studies. The lack of differential induction of IL-8 release or apoptosis in gastric epithelial cells by *hrgA*$^+$ strains compared with *hpy*IIIR strains does not support a direct role in virulence. It cannot be excluded that acquisition of *hrgA* is selected by gastric cancer devel-

opment, rather than being a causative factor. Whatever the case, the important clinical point is that *hrgA* may represent a novel marker for individuals with gastric cancer in Asia, where no such discriminatory *H. pylori* markers exist at present. The mere presence of *hrgA* may not be the only relevant factor for gastric cancer. Disease risk also could depend on transcription and/or translation of the *hrgA* gene product or on indirect effects thereof on expression of other genes. However, these findings provide a framework for future studies that can more mechanistically delineate the role of this locus in gastric carcinogenesis. Further studies are necessary to examine the function of *hrgA* and to ascertain its correlation with clinical outcome. If this observation is confirmed, *hrgA* may be used in the future to identify individuals of higher gastric cancer risk.

ACKNOWLEDGMENTS

We thank Dr. Sung-Kook Kim for providing bacterial strains.

REFERENCES

1. Blaser, M. J. Hypothesis: the changing relationships of *Helicobacter pylori* and humans: implications for health and disease. J. Infect. Dis., *179:* 1523–1530, 1999.
2. Goossens, H., Glupczynski, Y., Burette, A., Lambert, J. P., Vlaes, L., and Butzler, J. P. Role of the vacuolating toxin from *Helicobacter pylori* in the pathogenesis of duodenal and gastric ulcer. Med. Microbiol. Lett., *1:* 153–159, 1992.
3. Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., Figura, N., and Rappuoli, R. 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, 1993.
4. Crabtree, J. E., and Farmery, S. M. *Helicobacter pylori* and gastric mucosal cytokines: evidence that CagA-positive strains are more virulent. Lab. Investig., *73:* 742–745, 1995.
5. Blaser, M. J., Perez-Perez, G. I., Kleanthous, H., Cover, T. L., Peek, R. M., Chyou, P. H., Stemmermann, G. N., and Nomura, A. Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res., *55:* 2111–2115, 1995.
6. Cover, T. L., Glupczynski, Y., Lage, A. P., Burette, A., Tummuru, M. K., Perez-Perez, G. I., and Blaser, M. J. Serologic detection of infection with *cagA*$^+$ *Helicobacter pylori* strains. J. Clin. Microbiol., *33:* 1496–1500, 1995.
7. Maeda, S., Ogura, K., Yoshida, H., Kanai, F., Ikenoue, T., Kato, N., Shiratori, Y., and Omata, M. Major virulence factors, VacA and CagA, are commonly positive in *Helicobacter pylori* isolates in Japan. Gut, *42:* 338–343, 1998.
8. Sadakane, Y., Kusaba, K., Nagasawa, Z., Tanabe, I., Kuroki, S., and Tadano, J. Prevalence and genetic diversity of *cagD*, *cagE*, and *vacA* in *Helicobacter pylori* strains isolated from Japanese patients. Scand. J. Gastroenterol., *34:* 981–986, 1999.
9. Zheng, P. Y., Hua, J., Yeoh, K. G., and Ho, B. Association of peptic ulcer with increased expression of Lewis antigens but not *cagA*, *iceA*, and *vacA* in *Helicobacter pylori* isolates in an Asian population. Gut, *47:* 18–22, 2000.
10. Xu, Q., Morgan, R. D., Roberts, R. J., and Blaser, M. J. Identification of type II restriction and modification systems in *Helicobacter pylori* reveals their substantial diversity among strains. Proc. Natl. Acad. Sci. USA, *97:* 9671–9676, 2000.
11. Tomb, J. F., White, O., Kerlavage, A., Clayton, R. A., Sutton, G. G., Fleischmann, R. D., Ketchum, K. A., Klenk, H. P., Gill, S., Dougherty, B. A., Nelson, K., Quackenbush, J., Zhou, L., Kirkness, E. F., Peterson, S., Loftus, B., Richardson, D., Dodson, R., Khalak, H. G., Glodek, A., McKenney, K., Fitzegerald, L. M., Lee, N., Adams, M. D., Hickey, E. K., Berg, D. E., Gocayne, J. D., Fujii, C., Bowman, C., Watthey, L., Walin, E., Hayes, W. S., Borodorsky, M., Karp, P. D., Smith, H. O., Fraser, C. M., and Venter, J. C., The complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature (Lond.), *388:* 539–547, 1997.
12. Alm, R. A., and Trust, T. J. Analysis of the genetic diversity of *Helicobacter pylori*: the role of two genomes. J. Mol. Med., *77:* 834–846, 1999.
13. Kong, H., Lin, L. F., Porter, N., Stickel, S., Byrd, D., Posfai, J., and Roberts, R. J. Functional analysis of putative restriction-modification system genes in the *Helicobacter pylori* J99 genome. Nucleic Acids Res., *28:* 3216–3223, 2000.
14. Lin, L. F. Posfai, J., Roberts, R. J., and Kong, H. Comparative genomics of the restriction-modification systems in *Helicobacter pylori*. Proc. Natl. Acad. Sci. USA, *98:* 2740–2745, 2001.
15. Vitkute, J., Stankevicius, K., Tamulaitiene, G., Maneliene, Z., Timinskas, A., Berg, D. E., and Janulaitis, A. Specificities of eleven different DNA methyltransferases of *Helicobacter pylori* strain 26695. J. Bacteriol., *183:* 443–450, 2001.
16. Bennett, S. P., and Halford, S. E. Recognition of DNA by type II restriction enzymes. Curr. Top. Cell. Regul., *30:* 57–104, 1989.
17. Bickle, T. A., and Kruger, D. H. Biology of DNA restriction. Microb. Rev., *57:* 434–450, 1993.
18. Pingoud, A., and Jeltsch, A. Recognition and cleavage of DNA by type-II restriction endonucleases. Eur. J. Biochem., *246:* 1–22, 1997.
19. Ueno, T., Ito, H., Kimizuka, F., Kotani, H., and Nakajima, K. Gene structure and expression of the *Mbo*I restriction-modification system. Nucleic Acids Res., *21:* 2309–2313, 1993.
20. Ando, T., Xu, Q., Torres, M., Kusugami, K., Israel, D. A., and Blaser, M. J. Restriction-modification system differences in *Helicobacter pylori* are a barrier to interstrain plasmid transfer. Mol. Microbiol., *37:* 1052–1065, 2000.
21. Peek, R. M., Thompson, S. A., Donahue, J. P., Tham, K. T., Atherton, J. C., Blaser, M. J., and Miller, G. G. Adherence to gastric epithelial cells induces expression of a *Helicobacter pylori* gene, *iceA*, that is associated with clinical outcome. Proc. Assoc. Am. Physicians, *110:* 531–544, 1998.
22. van Doorn, L. J., Figueiredo, C., Sanna, R., Plaisier, A., Schneeberger, P., de Boer, W., and Quint, W. Clinical relevance of the *cagA*, *vacA*, and *iceA* status of *Helicobacter pylori*. Gastroenterology, *115:* 58–66, 1998.
23. Kidd, M., Peek, R. M., Lastovica, A. J., Israel, D. A., Kummer, A. F., Louw, J. A. Analysis of *iceA* genotypes in South African *Helicobacter pylori* strains and relationship to clinically significant disease. Gut, *49:* 629–635, 2001.
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
25. Wilson, K. Preparation of genomic DNA from bacteria. *In:* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (eds.), Current Protocols in Molecular Biology, Vol. 1, pp. 2.4.1–2.4.5. New York: John Wiley & Sons, 1995.
26. Wang, Y., and Taylor, D. E. Chloramphenicol resistance in *Campylobacter coli*. Gene, *94:* 23–28, 1990.
27. Ando, T., Kusugami, K., Ohsuga, M., Shinoda, M., Sakakibara, M., Saito, H., Fukatsu, A., Ichiyama, S., and Ohta, M. Interleukin-8 activity correlates with histological severity in *Helicobacter pylori*-associated antral gastritis. Am. J. Gastroenterol., *91:* 1150–1156, 1996.
28. Figueiredo, C., Quint, W. G., Sanna, R., Sablon, E., Donahue, J. P., Xu, Q., Miller, G. G., Peek, R. M., Blaser, M. J., and van Doorn, L. J. Genetic organization and heterogeneity of the *iceA* locus of *Helicobacter pylori*. Gene, *246:* 59–68, 2000.
29. Xu, Q., and Blaser, M. J. Promoters of the CATG-specific methyltransferase gene *hpyIM* differ between *iceA1* and *iceA2 Helicobacter pylori* strains. J. Bacteriol., *183:* 3875–3884, 2001.
30. Xu, Q., Peek, R. M., Miller, G. G., and Blaser, M. J. The *Helicobacter pylori* genome is modified at CATG by the product of *hpy*IM. J. Bacteriol., *179:* 6807–6815, 1997.
31. Miehlke, S., Kirsch, C., Agha-Amiri, K., Gunther, T., Lehn, N., Malfertheiner, P., Stolte, M., Ehninger, G., and Bayerdorffer, E. The *Helicobacter pylori vacA* s1, m1 genotype and *cagA* is associated with gastric carcinoma in Germany. Int. J. Cancer, *87:* 322–327, 2000.
32. Van Doorn, L. J., Figueiredo, C., Megraud, F., Pena, S., Midolo, P., Queiroz, D. M., Carneiro, F., Vanderborght, B., Pegado, M. D., Sanna, R., De Boer, W., Schneeberger, P. M., Correa, P., Ng, E. K., Atherton, J., Blaser, M. J., and Quint, W. G. Geographic distribution of *vacA* allelic types of *Helicobacter pylori*. Gastroenterology, *116:* 823–830, 1999.

A *Helicobacter pylori* Restriction Endonuclease-replacing Gene, *hrgA*, Is Associated with Gastric Cancer in Asian Strains

Takafumi Ando, Trudy M. Wassenaar, Richard M. Peek, Jr., et al.

Cancer Res 2002;62:2385-2389.