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Analysis and Typing of the *vacA* Gene from *cagA*-Positive Strains of *Helicobacter pylori* Isolated in Japan

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Approximately 50% of *Helicobacter pylori* strains produce a cytotoxin that is encoded by *vacA* and that induces vacuolation of eukaryotic cells. Mosaicism in *vacA* alleles was reported, and there are three different families of *vacA* signal sequences (s1a, s1b, and s2) and two different families of middle-region alleles (m1 and m2). In addition, the *vacA* genotype of a strain is associated with its cytotoxin phenotype and its capacity to induce peptic ulceration. To clarify the strain diversity of *H. pylori* in Japan, 87 Japanese clinical isolates of *H. pylori* (40 from patients with chronic atrophic gastritis, 25 from patients with duodenal ulcer, 16 from patients with gastric ulcer, 3 from patients with both duodenal and gastric ulcers, and 3 from patients with intestinal type gastric cancer) were characterized by *vacA* typing by PCR and DNA sequencing. Eighty-four of the 87 isolates were s1a/m1, one was s1b/m1, and two could not be typed. Moreover, all isolates in this study were *cagA* positive. There were no distinct differences between the cytotoxin-producing strains and cytotoxin-nonproducing strains within the 0.73-kb middle region. Japanese strains were highly homologous, with more than 96% identity in this region, in which maximum divergence has been reported. In addition, there were no associations between the specific *vacA* types and the level of in vitro cytotoxin activity or the clinical consequences. These results indicate that the *cagA*-positive, s1a/m1-type strains are common in Japan, regardless of the *vacA* phenotype or clinical outcome.

Helicobacter pylori is the major causative agent of chronic active gastritis in humans, and infection with this organism is an important etiological factor in the pathogenesis of peptic ulcer and gastric cancer (2). However, most people harboring *H. pylori* are asymptomatic, and only a few patients infected with this bacterium develop peptic ulcer or gastric cancer. One possible explanation for this phenomenon is that patients with serious gastroduodenal lesions are infected by virulent *H. pylori* strains, whereas those with simple chronic gastritis and no ulcer or cancer are infected by organisms with low pathogenic potential. One important virulence factor is a vacuolating cytotoxin that induces the formation of intracellular vacuoles in eukaryotic cells in vitro (5, 11, 18). Although the gene that encodes the cytotoxin, designated *vacA*, is present in nearly all strains (6, 22, 25, 30), only about 50% of *H. pylori* strains can produce detectable amounts of this cytotoxin (5). Cytotoxic strains have been detected more frequently only among patients with peptic ulcer than those with chronic gastritis (9, 29). In addition, experimental intragastric administration of the purified cytotoxin to mice resulted in epithelial damage and mucosal ulceration (30). These results confirmed the virulence potential of cytotoxin and its close association with peptic ulceration.

A recent study by Atherton et al. (1) focused on the differences between a cytotoxin-producing (tox^+) strain and cytotoxin-nonproducing (tox^-) strain, and this indicated the existence of three different families of the *vacA* signal sequence (s1a, s1b, and s2) and two different families of the *vacA* middle-region allele (m1 and m2). The *vacA* genotype of a strain is closely associated with its cytotoxin phenotype, *cagA* status, and capacity to induce peptic ulceration.

The prevalence of both gastric cancer and atrophic gastritis is extremely high in Japan (17, 21, 24). We have reported previously that tox^+ *H. pylori* isolates were more prevalent in patients with severe atrophic gastritis and that the cytotoxin activities in *H. pylori* isolates from patients with severe atrophic gastritis were much higher than those from patients with mild atrophic gastritis in Japan (19). The lineage of *H. pylori* isolates infecting Japanese subjects may be different from that of isolates in other parts of the world or a specific strain may have accumulated in the Japanese population.

In this study, the *vacA* region of *H. pylori* was characterized by PCR typing and direct sequencing to clarify *H. pylori* strain diversity in Japan.

MATERIALS AND METHODS

Patients. Clinical isolates of *H. pylori* were obtained from 87 patients during gastroduodenal endoscopy in the Second Department of Internal Medicine, Fukui Medical School, Fukui, Japan. None of the patients had received nonsteroidal anti-inflammatory drugs or antacids, and none had been prescribed antibiotics recently. The diagnosis was made endoscopically by two endoscopists. Twenty-five subjects (mean ± standard deviation [SD] age, 46.9 ± 13.5 years) had duodenal ulcer, 16 (mean age, 62.8 ± 16.2 years) had gastric ulcer, 3 (mean age, 52.0 ± 23.1 years) had both duodenal and gastric ulcers, and 3 (mean age, 60.0 ± 12.8 years) had intestinal-type gastric adenocarcinoma. Forty had chronic atrophic gastritis and were subdivided into two groups according to the grade of atrophy. The grade of atrophy was diagnosed endoscopically by using the criteria of Kimura and Takemoto (14) reported previously. The histological atrophy of the antrum and the endoscopic atrophy assessed by these criteria are positively correlated (12). Atrophy with patterns C-I and C-II was defined as mild, while atrophy with patterns C-III, O-I, O-II, and O-III were defined as severe (19). Of 40 patients with chronic atrophic gastritis, 17 (mean age, 49.4 ± 13.5 years) had mild atrophy. In contrast, the remaining 23 patients (mean age, 65.7 ± 9.85 years) had severe atrophy.

Isolation and culture of *H. pylori*. Two gastric biopsy specimens for culture were sequentially taken from the gastric body and antrum by using a sterilized endoscope and were then inoculated onto Trypticase Soy Agar-II containing 5%

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TABLE 1. Primers used for *vacA* typing and homology analysis

Region	Primer	Nucleotide sequence	Location of PCR product	Reference or source
m1	VA3-F	5′ GGTCAAAATGCGGTCATGG 3′	2741–3030 (290 bp)	1
	VA3-R	5′ CCATTGGTACCTGTAGAAAC 3′		
m2	VA4-F	5′ GGAGCCCCAGGAAACATTG 3′	976–1327 (352 bp)	1
	VA4-R	5′ CATAACTAGCGCCTTGCAC 3′		
s1a	SS1-F	5′ GTCAGCATCACACCGCAAC 3′	866–1055 (190 bp)	1
	VA1-R	5′ CTGCTTGAATGCGCCAAAC 3′		
s1b	SS3-F	5′ AGCGCCATACCGCAAGAG 3′	869–1055 (187 bp)	1[a]
	VA1-R	5′ CTGCTTGAATGCGCCAAAC 3′		
s2	SS2-F	5′ GCTTAACACGCCAAATGATCC 3′	371–569 (199 bp)	1[b]
	VA1-R	5′ CTGCTTGAATGCGCCAAAC 3′		
Middle	MID-F	5′ CCTTGGAATTATTTTGACGCTAG 3′	2621–3091 (471 bp)	This study[a]
	MID-R	5′ GTTGTTATAAAGGGCTAGGCGCTC 3′		
Signal	SIG-F	5′ ATGGAAATACAACAAACACACCG 3′	798–1134 (337 bp)	This study[a]
	SIG-R	5′ CAACCTTCCATCAATCTTACTGGA 3′		
1.3-kb region	VAS9-F	5′ CGACATTAGTTTGGGAAGATTTGT 3′	2260–3552 (1,293 bp)	This study[a]
	VAS6-R	5′ CCCGCATCATGGCTATCAATCAAT 3′		
	VAS8-R	5′ AGATCTTGAGCGCTGTTAATCTTG 3′	2932–2955	This study[a,c]
	VAS7-F	5′ AGGCAATGCAGCAGCTATGATGTT 3′	2863–2886	This study[a,c]
	VAS7mod-F	5′ AGGCAATGCAGCAGTCATGAGTTT 3′	2863–2886	This study[a,c]

[a] Corresponding to the nucleotide position of *H. pylori* 60190.
[b] Corresponding to nucleotide position of *H. pylori* 87-203.
[c] Primer used for only DNA sequencing.

sheep blood (TSA-II) plates (Nippon Becton Dickinson, Tokyo, Japan), and the plates were incubated for 5 days at 37°C under microaerobic conditions (O_2, 5%; CO_2, 15%; N_2, 80%). Then, 3-day liquid cultures of *H. pylori* were obtained by inoculating a few colonies from those TSA-II plates into 20 ml of brucella broth supplemented with 10% fetal calf serum. The bacterial suspensions were centrifuged at 1,300 × *g* for 10 min, and the supernatants were used for measurement of cytotoxin activity. Chromosomal DNA was extracted from the pellets by the protease and phenol-chloroform method, suspended in 300 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA), and stored at 4°C until PCR amplification.

Evaluation of cytotoxin production and quantification of activity. A431 (ATCC CRL 1555) cells derived from a human squamous cell carcinoma were kindly provided by N. Shimizu (Keio University, Tokyo, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (IBL, Gunma, Japan) and kanamycin (100 μg/ml) in 5% CO_2 and 100% humidity at 37°C. A431 cells (0.2×10^4 cells) were inoculated into each well of 96-well microtiter plates. After a 24-h incubation, the medium was replaced with 90 μl of fresh medium supplemented with 10 mmol of ammonium chloride per liter to potentiate cytotoxin activity. The supernatants of *H. pylori* liquid culture were concentrated 20-fold by using 100-kDa ultrafiltration membranes (Ultrafree C3HK; Millipore, Bedford, Mass.) and were then sterilized by passage through 0.22-μm-pore-size filters (Ultrafree G3GV; Millipore). These concentrated culture supernatants were serially diluted twofold (1:1 to 1:128) with A431 culture medium, and dilutions were scored from 1 (1:1) to 8 (1:128). Ten-microliter aliquots were added to 90 μl of A431 culture medium in 96-well plates. After a 24-h incubation, cells were examined microscopically for the presence of intracellular vacuolization. When more than 50% of the cells displayed vacuolization, the isolate was defined as cytotoxin positive. The maximum dilution of concentrated supernatant that produced vacuolization was defined as the score of cytotoxin activity (1 to 8). We defined a high grade of cytotoxin activity as a score of ≥4 and a low grade of cytotoxin activity as a score of between 1 and 3. During the assay, broth culture supernatant without *H. pylori* inoculation was used as a negative control.

Primers. On the basis of published sequences (1, 6), oligonucleotides were prepared to amplify the *vacA* signal and the middle region. For DNA sequencing for *vacA* typing and homology analysis, eight primers were also synthesized. The primers used in this study are listed in Table 1.

PCR-based typing of *vacA* homolog. To type the *vacA* signal and middle region, PCR was performed in 50-μl reaction mixtures containing 100 ng of genomic DNA, 250 nM (each) primer, 1× reaction buffer, 1.5 mM $MgCl_2$, 1 U of AmpliTaq DNA polymerase, and distilled, sterilized water in the GeneAmp PCR system 2400 (Perkin-Elmer Japan, Chiba, Japan). After boiling at 94°C for 5 min, amplification was carried out for 27 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s; the mixture was then cycled at 72°C for 7 min to complete the elongation step and was finally stored at 4°C. PCR products were separated by 2% agarose gel electrophoresis and examined under UV illumination.

DNA sequencing for homology analysis of *vacA* middle region. Forty-four isolates (17 high-grade tox^+, 14 low-grade tox^+, 13 tox^-) were randomly selected for homology analysis. To determine the nucleotide sequences of the *vacA* middle region, a 1.3-kb region encoded by a partial *vacA* open reading frame (corresponding to nucleotides 2244 to 3553 of *H. pylori* 60190 *vacA*) was amplified with two specific primers, VAS-9F and VAS-6R, as described in Table 1. PCR conditions were as follows: heating at 94°C for 5 min, followed by 25 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. After the tubes were held at 72°C for 7 min, they were stored at 4°C. The PCR products were then purified by using Centricon-100 Concentrator columns (Amicon, Beverly, Mass.). DNA sequencing was performed by the dideoxynucleotide chain termination method with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer Japan) in an automated DNA sequencer (model 373A or model 310; Perkin-Elmer Japan). According to the manufacturer's protocol, reagent mixtures containing 5 μl of purified PCR product, 3.2 pmol of primer, 8 μl of Terminator Ready Reaction Mix, and 5 μl of sterilized distilled water were prepared. Reaction tubes were placed in the thermal cycler, and thermal cycling was started under the following conditions: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, which was repeated for 25 cycles. Cycle sequencing reactions were performed on both DNA strands by using each of the four primers (primers VAS9-F, VAS6-R, VAS7-F, and VAS8-R) and the 1.3-kb PCR product as a template.

Homology analysis of the *vacA* middle region. The nucleotide sequence of the 0.73-kb *vacA* middle region (corresponding to amino acid residues 509 to 752 of the *H. pylori* 60190 *vacA* product) was constructed from the sequence data of the 1.3-kb PCR product. Nucleotide sequence homology within the 0.73-kb *vacA* middle region was analyzed with GENETYX-MAC, version 7.3, software (Software Development Co., Tokyo, Japan). Strains F84, F71, and F94 were selected as the representative Japanese strains of high-grade tox^+, low-grade tox^+, and tox^-, respectively. The sequences of strains 60190 (tox^+) and 87-203 (tox^-) were also selected as standards (the sequences were from the GenBank sequence data library; accession numbers U05676 and U05677, respectively). The nucleotide sequence homology and the deduced amino acid sequence homology between each of these five strains (strains F84, F71, F94, 60190, and 87-203) and the three groups of strains (17 high-grade tox^+, 14 low-grade tox^+ and 13 tox^- strains) were determined as the mean ± SD identity.

Detection of the *cagA* gene. To determine whether the presence of *cagA* is associated with the s1 *vacA* signal sequence, the *cagA* gene was detected by Southern blot hybridization. Genomic DNA (1 μg) was digested with *Hin*dIII (Toyobo, Tokyo, Japan) according to the manufacturer's instructions and was subjected to electrophoretic separation in 1% agarose gels. Genomic DNA was denaturated, transferred onto nitrocellulose (27), and prehybridized at 42°C for 3 h in 50% formamide–25 mM sodium phosphate (pH 6.5)–500 μg sonicated salmon testis DNA per ml–5× Denhardt's solution. Filters were hybridized overnight in a solution containing 50% formamide, 10% dextran sulfate, 20 mM sodium phosphate (pH 6.5), 250 μg of sonicated salmon testis DNA per ml, 2× Denhardt's solution, and *cagA* probe labeled with [^{33}P]dCTP by the random primer method as described previously (7). The *cagA* probe was a 320-bp PCR product (corresponding to nucleotides 1764 to 2083 of *H. pylori* CCUG 17874 *cagA* [3]) amplified by two *cagA*-specific primers (5′-AGACAACTTGAGCGA GAAAG-3′ and 5′-TATTGGGATTCTTGGAGGCG-3′). PCR was performed as described in the section on PCR-based typing of the *vacA* homolog at a modified annealing temperature of 55°C. The filters were rinsed several times at room temperature in a solution containing 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.05% sodium dodecyl sulfate to remove excess hybridization solution and were then washed at 52°C for 10 min in the same solution (0.1× SSC and 0.05% sodium dodecyl sulfate).

RESULTS

Typing of the *vacA* gene. Typing of the *vacA* gene of 87 clinical isolates was performed by PCR as described previously (1). Eighty-one of the 87 isolates (93.1%) were s1a and 1 isolate (1.1%) was s1b. The *vacA* signal sequences of five strains (5.7%) could not be typed. With regard to the middle region, only three strains (3.4%) were found to have the m1 allele type by PCR. The remaining strains could not be assigned to either the m1 or the m2 allele type. To complete the *vacA* typing, the signal sequence genotypes of 5 strains and the middle region genotypes of 84 strains were determined by DNA sequencing. Two sets of primers (SIG-F-SIG-R and MID-F-MID-R) were used to sequence the two regions (signal sequence [SIG] and middle region [MID]), including the signal (s1a, s1b, and s2) and the middle (m1 and m2) regions. DNA sequencing revealed mutations within primer regions, which caused unsuccessful PCR typing of the *vacA* gene (data not shown). Overall, 84 strains (96.6%) were s1a/m1, 1 strain (1.1%) was s1b/m1, and the types of the remaining 2 strains (2.3%) could not be determined because of a 61-bp insertion in the signal region and unsuccessful PCR of the middle region for sequencing. There were no associations between the specific *vacA* types and clinical outcome or the severity of mucosal atrophy.

Association of *cagA* positivity with s1 signal sequence. The presence of the *cagA* gene was investigated in all 87 isolates by Southern blotting. All 87 isolates possessed the *cagA* gene (Fig. 1). This result suggested the close association between *cagA* status and the s1 signal sequence. However, we could not confirm its significance because of the lack of both *cagA*-negative strains and s2 type strains in our isolates (by Fisher's exact probability test, $P = 1.0$).

DNA sequencing for homology analysis of the *vacA* middle region. To compare the homology among strains, the *vacA* PCR products of 44 randomly selected isolates were sequenced. The 1.3-kb PCR product (corresponding to nucleotides 2244 to 3553 of the *H. pylori* 60190 *vacA* gene) was amplified by VAS9-F and VAS6-R as indicated in Table 1 and was used as a template. The sequence of the VAS7-F region of almost all of our isolates differed by four or five nucleotides from those of previously reported strains (data not shown). Thus, a modified VAS7-F primer (named VAS7mod-F) corresponding to our isolates was constructed, and the isolates were successfully sequenced. From the sequencing data obtained with four primers (primers VAS9-F, VAS6-R, VAS8-R, and either VAS7-F or VAS7mod-F), the nucleotide sequences of the 0.73-kb *vacA* middle region (corresponding to amino acid residues 509 to 752 of the *H. pylori* 60190 *vacA* product) were determined. No insertions or deletions were found within this 0.73-kb middle region among our 44 isolates, and the deduced amino acid sequences could be determined without a stop codon (data not shown). To analyze divergence within the *vacA* middle region, the nucleotide and deduced amino acid sequence identities were calculated and compared. Table 2 shows the nucleotide and amino acid sequence identities among the five strains (F84, F71, F94, 60190, and 87-203) and three groups of Japanese isolates (17 high-grade tox$^+$ strains, 14 low-grade tox$^+$ strains and 13 tox$^-$ strains). The sequences of the Japanese isolates were highly homologous (more than 96% identity) with one another, regardless of the *vacA* phenotype.

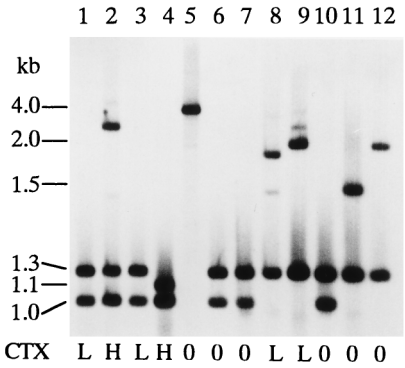

FIG. 1. Southern blot of chromosomal DNA from 12 strains (lanes 1 through 12, respectively) cut with *Hin*dIII. Restriction fragments were hybridized with the *cagA* probe. The *cagA* gene is present in all isolates. There are several restriction fragment length polymorphism patterns, but no specific pattern for cytotoxity was identified. Size markers are shown on the left. Abbreviations: CTX, cytotoxin activity; H, high-grade tox$^+$; L, low-grade tox$^+$; 0; tox$^-$.

Three strains (strains F37, F79, and F80) that were determined to be type m1 by PCR had slightly lower degrees of sequence homology. The sequence homologies of eight strains (strains F37, F79, F80, F84, F71, F94, 60190, and 87-203) are presented in Table 3. Although strains F37 and F79 were

TABLE 2. Homology analysis of 0.73-kb *vacA* middle region[a]

Comparison		% Identity (mean ± SD)	
Test strain	Randomly selected isolates	Nucleotide	Amino acid
F84 (high tox$^+$)	16 high-grade tox$^+$	96.9 ± 2.8	97.6 ± 3.6
	14 low-grade tox$^+$	97.1 ± 2.7	97.6 ± 3.6
	13 tox$^-$	97.6 ± 0.6	98.1 ± 0.9
F71 (low tox$^+$)	17 high-grade tox$^+$	97.2 ± 2.8	97.6 ± 3.7
	13 low-grade tox$^+$	97.4 ± 3.0	97.1 ± 4.0
	13 tox$^-$	97.8 ± 0.6	98.1 ± 0.8
F94 (tox$^-$)	17 high-grade tox$^+$	97.3 ± 2.9	97.3 ± 3.6
	14 low-grade tox$^+$	97.6 ± 2.7	97.1 ± 4.0
	12 tox$^-$	97.7 ± 0.5	97.8 ± 0.7
60190 (tox$^+$)	17 high-grade tox$^+$	89.4 ± 2.4	86.9 ± 3.3
	14 low-grade tox$^+$	89.3 ± 2.6	86.9 ± 3.4
	13 tox$^-$	88.7 ± 0.3	86.1 ± 0.8
87-203 (tox$^-$)	17 high-grade tox$^+$	72.6 ± 0.3	57.0 ± 0.6
	14 low-grade tox$^+$	72.6 ± 0.4	56.9 ± 0.6
	13 tox$^-$	72.8 ± 0.4	57.0 ± 0.5

[a] GenBank sequence accession nos. for strains F84, F71, F94, 60190, and 87-203 are U91575, U91576, U91577, U05676, and U05677, respectively. The 0.73-kb *vacA* middle region corresponds to nucleotides 2321 to 3052 of *H. pylori* 60190 *vacA*.

TABLE 3. Analysis of divergence within a 0.73-kb middle region of *H. pylori vacA*[a]

Strain	% Identity[b]							
	60190	F37	F79	F80	F84	F71	F94	87-203
60190 (tox$^+$)		98.1	98.0	92.1	88.6	88.2	89.0	73.2
F37 (low-grade tox$^+$)	98.9		99.9	92.1	88.1	87.8	88.6	73.6
F79 (high-grade tox$^+$)	98.8	100		91.9	88.0	87.7	88.5	73.6
F80 (high-grade tox$^+$)	89.8	89.8	89.7		92.3	92.5	92.9	72.8
F84 (high-grade tox$^+$)	86.4	85.6	85.6	92.2		98.0	98.1	72.4
F71 (low-grade tox$^+$)	85.6	84.3	84.8	92.2	99.2		98.2	72.2
F94 (tox$^-$)	86.0	85.2	85.2	91.8	98.8	98.8		72.9
87-203 (tox$^-$)	59.0	58.5	58.5	58.5	57.8	57.8	57.8	

[a] Strains F37, F79, F80, F84, F71, and F94 are Japanese isolates used in this study. The GenBank sequence accession nos. for strains F37, F79, F80, F84, F71, F94, 60190, and 87-203 are U91578, U91579, U91580, U91575, U91576, U91577, U05676, and U05677, respectively.

[b] Numbers above the diagonal space are percent nucleotide sequence identity, and those below the diagonal space are percent amino acid sequence identity.

isolated in Japan, they were genetically closer to strain 60190, which was isolated from a patient in England (18), than the Japanese strains.

DISCUSSION

In this study, we showed that *H. pylori* isolates from Japan contained predominantly the s1a/m1 family of *vacA* alleles. Moreover, among the Japanese clinical isolates, there were no distinct differences in the 0.73 kb of the *vacA* middle region between tox$^+$ strains and tox$^-$ strains. Our results indicate considerably less diversity among the strains than those reported by Atherton et al. (1), which indicated that multiple *vacA* types exist and that the *vacA* signal sequence and middle region are markedly different between tox$^+$ strains and tox$^-$ strains. Their study also demonstrated that s1/m1 strains produced higher levels of vacuolating cytotoxin activity. Although almost all isolates examined in this study were of the s1/m1 type, the magnitude of the in vitro cytotoxin activity was not always high.

The reason why the s1a/m1 strains have accumulated in Japan is unclear. The *H. pylori* genome showed a high degree of genetic diversity among strains (28). Genomic rearrangements may be associated with the uptake of DNA by natural transformation (20, 36), as seen in *Campylobacter jejuni* and *Campylobacter coli* (35). We speculated that some factors required for genetic diversity may be different in Japan compared with those in the United States. The route of *H. pylori* infection remains controversial (8, 31), and no environmental niche has been found for *H. pylori*. Humans, some primates, and cats (10) represent the only significant reservoir. One hypothesis is that the low genetic heterogeneity in *vacA* of *H. pylori* isolated in Japan may be due to the traits of the country and people. Japan is a country consisting of a relatively homologous population and is geographically isolated. In addition, there has been little mixing with other ethnic groups throughout its history. Therefore, the opportunity for the transfer of DNA between strains of different genotypes may be lower than that in the United States.

Another hypothesis is that strains of the s1a/m1 *vacA* allele type may have a selective advantage and may adapt themselves to the gastric environment more effectively than those of other genotypes. The stomachs of Japanese patients tended to have a large area of atrophy and intestinal metaplasia, although gastritis may be localized to the antrum and there is much more multifocal atrophic gastritis in patients in the West (15, 16). Diffuse atrophy is considered to affect the colonization of *H. pylori* (13, 26). Although the simultaneous gastric infection with two *H. pylori* strains has been reported at frequencies of 10 to 13% (23), the PCR-restriction fragment length polymorphism patterns determined by using the *ureB* locus showed that almost all patients were infected with the same strain throughout their lifetimes (32). In addition, we have reported that cytotoxic strains were more frequently isolated from patients with severe atrophic gastritis (12, 19). Therefore, only s1/m1 strains may be able to survive in the low-acid gastric environment of the patients with chronic atrophic gastritis. It may explain the reason why s1a/m1 strains, which are considered to be the most cytotoxic, were frequently detected not only in patients with peptic ulcer but also in patients with chronic atrophic gastritis in Japan.

The reason why there were no distinct differences within the middle region between tox$^+$ strains and tox$^-$ strains is also unclear. Other factors within and outside the *vacA* gene must be investigated. Previous studies indicated a close association between the CagA protein (a 128-kDa cytotoxin-associated protein encoded by *cagA*) and vacuolating cytotoxin activity and concluded that the CagA protein regulates the production of vacuolating cytotoxin (3, 4, 33). Tummuru et al. (34), however, reported that disruption of the *cagA* gene by shuttle mutation did not influence the production of the vacuolating cytotoxin. In this study we found that all of our isolates were *cagA*-positive strains and indicated that the possession of *cagA* is not correlated with the grade of vacuolating cytotoxin activity.

In conclusion, among Japanese clinical isolates there was a clustering of the *cagA*-positive strains with the s1a/m1 family of *vacA* alleles, and no distinct differences within the signal sequence or the 0.73-kb middle region of *vacA* were detected between tox$^+$ and tox$^-$ strains. Another mechanism accounting for impaired vacuolating cytotoxin production may be revealed by analyzing the differences between tox$^+$ strains and tox$^-$ strains isolated from Japanese patients.

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