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Heterogeneity in the Activity of Mexican *Helicobacter pylori* Strains in Gastric Epithelial Cells and Its Association with Diversity in the *cagA* Gene▽†

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***Helicobacter pylori* CagA is translocated into gastric epithelial cells by a type IV secretion system and interacts with the Src homology 2 phosphatase, altering cell morphology. Multiple EPIYA motifs in CagA are associated with increased activity in cells and with gastric cancer. The aim of this work was to study the heterogeneity in activity in cells of multiple *H. pylori* single colonies isolated from a single patient and its association with polymorphism in *cagA*. The presence of *cagA*, *cagE*, *cagT*, and *cag10* was studied with 318 *H. pylori* isolates from the antra and corpora of 18 patients. AGS gastric epithelial cells were infected with 75 isolates, and interleukin-8 (IL-8) secretion, cytoskeletal changes, CagA translocation, and tyrosine phosphorylation were measured. The *cagA* 3′-variable region was sequenced for 30 isolates to determine the number and types of EPIYA motifs. Isolates from an individual stomach were usually genetically related and had quantitatively similar phenotypic effects on cells (IL-8 induction and cytoskeletal changes). However, strains from different patients with similar CagA EPIYA motif patterns varied widely in these phenotypes. Among isolates with an EPIYA-ABC pattern, the phenotype was variable: IL-8 induction ranged from 200 to 1,200 pg/ml, and morphological changes occurred in 20 to 70% of cells. In several cases, *cagA* sequence diversity appeared to explain the lack of CagA activity, as isolates with an EPIYA-ACC pattern or a modified B motif had reduced cell activity. *cag* pathogenicity island-positive *H. pylori* isolates displayed a high level of heterogeneity in the capacity to induce IL-8 secretion and morphological changes; an absent or modified B motif was associated with low activity.**

Helicobacter pylori infects up to 50% of the world's human population, lives for decades in the human stomach (17), and is associated with duodenal ulcers (16), gastric ulcers, distal gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma (13). Its ability to cause disease has been associated with the expression of several virulence factors, including the VacA cytotoxin (5, 6), BabA (19), the neutrophil-activating protein (HP-NAP) (30, 38), outer inflammatory protein A (OipA) (50), the duodenal ulcer-promoting gene (*dupA*) (28), and the *cag* pathogenicity island (*cag*PAI). The *cag*PAI is a 40-kb region originally acquired horizontally and inserted into the glutamate racemase gene (1, 15); it is present in about 50 to 60% of *H. pylori* isolates from Western countries and in >90% of isolates from East Asian countries. Some studies have shown that severe gastroduodenal diseases are associated with *H. pylori* strains that harbor an intact *cag*PAI (11, 22, 24, 26, 29, 32, 33), whereas other studies could not find a relationship (7, 25).

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Genes in the *cag*PAI encode a type IV secretion system (17) through which CagA is translocated into gastric epithelial cells, where it is phosphorylated by Src kinases on the tyrosine residues of a five-amino-acid (EPIYA) motif (10, 34, 40, 44). Once phosphorylated CagA interacts with Src homology 2 (SHP-2) phosphatase, it stimulates downstream signaling cascades involved in the reorganization of the cytoskeleton, resulting in cellular morphological changes such as the "hummingbird" phenotype, which is characterized by a prominent elongation and spreading of host cells, including the production of filopodia and lamellipodia (20, 39). Attachment of *H. pylori* to gastric epithelial cells also induces the production of interleukin-8 (IL-8), mobilizing inflammatory cells to the site of infection in a process involving NF-κB activation (36, 42, 43). Recently, some authors reported that IL-8 release might be mediated by mitogen-activated protein kinases through tyrosine phosphorylation of CagA (14, 27).

The *cagA* gene contains a 5′ end which is highly conserved and a 3′ end which is variable. The 3′-variable region contains several repeat sequences, each of which contains an EPIYA motif; the size variation in CagA correlates with the number of repeat sequences located in this region (47). EPIYA motifs in Western *H. pylori* isolates are classified as EPIYA-A, EPIYA-B, and EPIYA-C and are defined by the amino acid sequences surrounding the EPIYA motifs. It has been suggested that the number of EPIYA-C sites directly correlates with the level of tyrosine phosphorylation, SHP-2 binding ac-

TABLE 1. Specific primers used to identify *cag*PAI genes in this study

Gene	Primer	Primer sequence (5′→3′)	Product size (bp)	Reference
cagA (5′ end)	F1	GATAACAGGCAAGCTTTTTGAGG	349	47
	B1	CTGCAAAAGATTGTTTGGCAG	349	47
cagA (3′ end)	cag2	GGAACCCTAGTCGGTAATG	450–850	37
	cag4	ATCTTTGAGCTTGTCTATCG	450–850	37
cagE	101	TTGAAAACTTCAAGGATAGGATAGAGC	510	48
	102	GCCTAGCGTAATATCACCATTACCC	510	48
	PBRT-F	AAGGGTAAAGAAATGGGACTG	1,800	51
	PBRT-R	GGAAGCGTGATAAAAGAGCAATGT	1,800	51
cagT	cagT-F	ATGAAAGTGAGAGCAAGTGT	823	29
	cagT-R	TCACTTACCACTGAGCAAAC	823	29
	CAG13	TCTAAAAAGATTACGCTCATAGGCG	489	24
	CAG14	CTTTGGCTTGCATGTTCAAGTTGCC	489	24
cag10	cag10-F	ATGGAAGACTTTTTGTATAA	2,208	29
	cag10-R	TCACAGTTCGCTTGAACCCA	2,208	29
Empty site	2	ACATTTTGGCTAAATAAACGCTG	360	1
	25	TCATGCGAGCGGCGATGTG	360	1
RAPD-PCR	1254	CCGCAGCCAA		2
	1281	AACGCGCAAC		2

tivity, and cell damage. CagA proteins in East Asian *H. pylori* isolates possess EPIYA-A, EPIYA-B, and EPIYA-D motifs; the EPIYA-D motif has a higher affinity for SHP-2 than does the Western EPIYA-C motif and appears to induce more severe cellular changes (21).

Evidence showing that CagA proteins with more EPIYA motifs are more frequent in strains associated with cases of atrophic gastritis and gastric cancer than in strains from patients with chronic gastritis has been presented, suggesting an association between the size of the 3′-variable region of *cagA* and the clinical outcome (8, 9). In South Africa, *H. pylori* strains with four to six EPIYA motifs and higher CagA molecular weights were isolated from patients with gastric cancer (4). In addition, the study demonstrated that when *H. pylori* is cocultured with gastric epithelial cells, a correlation between the size of the CagA protein, the magnitude of tyrosine phosphorylation, and the intensity of cellular elongation exists (4). Similar results have been reported for strains from Chinese patients (51).

Several studies have addressed the correlation between genetic diversity in the *cag*PAI and disease, but few have reported on phenotypic diversity regarding the cellular activities of multiple isolates obtained from single patients. Accordingly, the aim of this work was to study phenotypic diversity in the activity in gastric epithelial cells of multiple *H. pylori* single colonies isolated from single patients and to correlate this diversity with the polymorphisms observed in the 3′-variable region of the *cagA* gene.

(Part of this work was submitted as a requirement for the D.Sc. degree of Adriana Reyes-Leon at Doctorado en Ciencias Biomedicas, Universidad Nacional Autonoma de Mexico.)

MATERIALS AND METHODS

Patients. We studied 10 children with chronic abdominal pain (seven boys and three girls) attending the Gastroenterology Department at the Hospital de Pediatria, Centro Medico Nacional Siglo XXI (CMN-SXXI), Instituto Mexicano del Seguro Social (IMSS), Mexico City, Mexico, with a mean age of 10.7 years (ranging from 7 to 16 years), and eight adults (five men and three women) attending the Gastroenterology Department at the Hospital de Especialidades, CMN-SXXI, IMSS, with a mean age of 57.2 years (ranging from 28 to 88 years). Three adult patients had gastric ulcers, four had duodenal ulcers, and one had nonulcer dyspepsia. Patients were subjected to endoscopy as part of the usual diagnostic protocol, and two biopsies each were taken from the antrum and corpus; one biopsy from each region was used to culture *H. pylori*. The study was approved by the ethics committee of the Hospital de Pediatria at CMN-SXXI, IMSS.

***Helicobacter pylori* culture and isolation of multiple single colonies from biopsies.** Antrum and corpus biopsy specimens were placed in sterile 0.9% saline solution, homogenized, and inoculated onto blood agar base (BBL, MD) plates supplemented with 5% sheep blood. The plates were incubated at 37°C in a 9% CO_2 atmosphere for up to 5 days. *H. pylori* was identified by colony and microscopic morphology and by positive oxidase, catalase, and urease tests. From each primary growth, 7 to 10 single colonies each were isolated from the antrum and corpus and propagated on blood agar medium. We studied a total of 200 isolates from children (mean of 20 per patient) and 118 isolates from adults (mean of 15 per patient).

Detection of *cag*PAI genes. DNAs were isolated from confluent plate cultures of each isolate, using the commercial Wizard method (Promega Corporation, Madison, WI) according to the manufacturer's instructions. DNAs from *H. pylori* strains 84-183 (ATCC 53726), 26695 (ATCC 700392), and Tx30a (ATCC 51932) were prepared for use as controls. The *cagE*, *cagT*, and *cag10* genes were selected for study because they are distributed along the *cag*PAI and because they are homologous to known genes in type IV secretion systems; in addition, *cagA* was studied because it encodes the protein which is translocated by this system. The presence of these genes was evaluated in the 318 *H. pylori* isolates by PCRs using specific primers (Table 1). Two sets of primers were used for each gene examined, except for the *cag10* gene (Table 1). All PCR mixtures consisted of 1 μl of chromosomal DNA template (100 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate (Boehringer Mannheim, Germany), 25 pmol of each primer, and 1.25 units of *Taq* DNA polymerase (Invitrogen, Life Technologies, Brazil) in a final volume of 25 μl. PCRs were performed in a thermal cycler (GeneAmp PCR system 9700; PE Applied Biosystems). Positive (strains 26695 and 84-183) and negative (strain Tx30a) controls for the *cag*PAI were included in each run. To test for the absence of the *cag*PAI, we used the *cag* empty-site assay with primers 2 and 25, which flank the left and right ends of the *cag*PAI (1). The PCR conditions for each reaction were described previously (1, 24, 29, 37, 47, 51). PCR products were electrophoresed, stained with ethidium bromide, and visualized under UV light.

To confirm the PCR results, dot blot hybridization was performed as follows. The PCR products for the *cagA*, *cagE*, *cagT*, and *cag10* genes were amplified with specific primer pairs F1 and B1, 101 and 102, cagT-F and cagT-R, and cag10-F and cag10-R, respectively, using chromosomal DNA from strain 84-183. PCR products were electrophoresed, and appropriate fragments (probes) were purified (Rapid Gel extraction system; Marligen Bioscience) and radioactively labeled with [α-^{32}P]dCTP by random primer extension (Megaprime DNA labeling system kit; Amersham Pharmacia Biotech). Hybridization was performed using Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech), using 20 ng of genomic DNA per spot for each isolate. Membranes were incubated with the probes, washed, and processed for autoradiography with X-ray film (Kodak).

RAPD-PCR method. Random amplified polymorphic DNA PCRs (RAPD-PCRs) were carried out in a 25-μl volume containing 20 ng of *H. pylori* genomic DNA, 2.5 μl of 10× PCR buffer, 3.5 mM $MgCl_2$, 20 pmol of primer 1254 or 1281 (Table 1), a 0.25 mM concentration of each deoxynucleoside triphosphate (Boehringer Mannheim, Germany), and 1.2 units of *Taq* DNA polymerase (Invitrogen, Life Technologies, Brazil), as previously reported (2). An aliquot of 20 μl of PCR product was electrophoresed in a 2% agarose gel at 80 V, stained, and visualized.

Sequencing of the 3′-variable region of the *cagA* gene. The 3′-variable region of *cagA* was amplified with primers cag2 and cag4 (37) (Table 1), and nucleotide sequencing was performed using the dideoxynucleotide chain termination method with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems), as previously described (8). Nucleotide and derived amino acid sequences were analyzed and aligned with the Chromas (version 1.62; Technelysium) and DNAMAN (version 3.0; Lynnon BioSoft) programs.

Biological activities of *H. pylori* on gastric epithelial cells. For translocation, phosphorylation, and IL-8 induction assays, the AGS cell line (ATCC CRL-1739) was grown in 75-cm^2 flasks with Ham's F-12 nutrient mixture (GIBCO, Invitrogen Corporation) supplemented with 10% heat-inactivated fetal bovine serum (F-12–10% FBS) (GIBCO, Invitrogen Corporation, USA) at 37°C in a 5% CO_2 atmosphere for 48 h. Next, serum-free F-12 medium was added, and cells were incubated for an additional 24 h. For cellular elongation assays, AGS cells (1×10^5/ml) were grown in six-well plates with F-12–10% FBS for 48 h. *H. pylori* strains to be tested were grown for 48 h in blood agar plates, and a single colony was reseeded on an agar plate and incubated for 24 h. *H. pylori* colonies were harvested and resuspended in serum-free F-12 medium to reach an optical density of 0.1 at 550 nm (1.2×10^8 bacteria/ml) before addition to AGS cells at a multiplicity of infection of 1:100. AGS cells in either 75-cm^2 flasks or six-well plates were cocultured with *H. pylori* isolates in serum-free F-12 medium for up to 48 h (see below). *H. pylori* strains 26695 and 60190 (ATCC 49503) were used as positive controls.

After coculture for 6 h, the cell culture supernatant was collected and stored at −80°C until it was tested for IL-8 induction. The amount of IL-8 in each sample was measured by an enzyme-linked immunosorbent assay, using an OptEIA human IL-8 kit (BD Biosciences) following the manufacturer's instructions. Cells were then washed twice with phosphate-buffered saline (PBS) containing 1 mM calcium chloride and 0.5 mM magnesium chloride, scraped from the flasks into 5 ml PBS containing 1 mM sodium vanadate, harvested by centrifugation at 1,000 × *g* for 10 min, and resuspended in 100 μl PBS-sodium vanadate and 50 μl 4× sample loading buffer (0.2 M Tris-HCl, pH 6.8, 0.4 M dithiothreitol, 8% sodium dodecyl sulfate, 40% glycerol, 0.4% bromophenol blue). Samples were boiled and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were separated in 9% polyacrylamide gels, electrotransferred to nitrocellulose membranes by standard procedures, and examined for the presence of the CagA protein by using a polyclonal antibody (1:1,000) (bN-20; Santa Cruz Biotechnology). Phosphorylated tyrosine was detected with a monoclonal antibody (1:3,000) (PY99; Santa Cruz Biotechnology). Blots were then incubated with horseradish peroxidase-conjugated rabbit anti-goat and goat anti-mouse antibodies (Zymed Laboratories, CA) and developed with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). In some cases, translocated CagA was detected using sera from infected patients (1:50 dilution in PBS) with high levels of anti-CagA antibodies, incubated with a peroxidase-conjugated goat antibody to human immunoglobulin G (Zymed Laboratories, CA) (1:1,000 dilution), and developed with ECL Western blotting detection reagents (18).

For induction of cellular elongation of AGS cells, AGS cells infected with *H. pylori* isolates were incubated for up to 48 h. Cells were then examined for cellular elongation by light microscopy (magnification, ×20), reading three randomly chosen fields (4); results were reported as percentages of cells exhibiting the cellular elongation effect.

Each isolate was tested for IL-8 induction, CagA translocation and phosphorylation, and cellular elongation in two separate experiments, with each sample run in duplicate in each assay.

***H. pylori* adherence and viability during coinfection.** To assess *H. pylori* adherence to AGS cells, coinfections were monitored by light microscopy (×40) during different periods to visualize bacteria attached to cells and bacterial mobility. The viability of the inoculated bacteria was monitored 1, 24, and 48 h after coinfection; for this purpose, an aliquot of 10 μl of a 1:100 dilution of the cell culture supernatant was taken, spread on blood agar plates, and cultured for 48 h.

Statistical analysis. A two-tailed Student *t* test was used to assess the relationship of *cag*PAI$^+$ and *cag*PAI$^-$ strains to induction of IL-8 release and cellular elongation in AGS cells. A *P* value of <0.05 (two-sided) was considered statistically significant.

Nucleotide sequence accession numbers. Nucleotide sequences were deposited in GenBank under accession numbers EF552407 to EF552424.

RESULTS

Presence of *cagA*, *cagE*, *cagT* and *cag10* genes. Among the 318 *H. pylori* isolates studied, 250 isolates (78.6%) had all four *cag*PAI genes (*cagA*, *cagE*, *cagT*, and *cag10*) (*cag*PAI$^+$) and 48 isolates (15.1%), which were positive for the PCR empty site, did not have the *cag*PAI genes (*cag*PAI$^-$ isolates) (Table 2). In all 20 isolates from patient 365, *cagA* was absent, but *cagE*, *cagT*, and *cag10* were present, indicating a partial deletion in the *cag*PAI. Patient 259 was colonized with both *cag*PAI$^+$ and *cag*PAI$^-$ isolates, which had different RAPD patterns (data not shown), showing evidence of a mixed infection (Table 2).

The 3′-variable region of the *cagA* gene was amplified from the 250 *cagA*-carrying isolates. There was diversity in the size of the fragment among isolates, with PCR products showing lengths of between 500 and 850 bp (Table 2). Although in the majority of cases all isolates from a patient showed the same length, in three cases (patients 555, 259, and 261) isolates presented different sizes for the *cagA* 3′-variable region. For patient 555, 19 isolates had a *cagA* fragment size of 550 bp and one had a size of 650 bp. Patient 261 had one isolate with a *cagA* fragment of 800 bp, whereas all others had a size of 550 bp (Table 2 and Fig. 1); analysis by RAPD-PCR showed that this variation was due to infection with multiple *H. pylori* strains. For patient 259, most isolates had a fragment size of 550 bp and two isolates had a size of 500 bp (Table 2 and Fig. 1); RAPD-PCR analysis showed that these isolates were of the same strain, with polymorphic variation in the 3′ region.

Diversity in IL-8 secretion by AGS cells cocultured with *cag*PAI$^+$ and *cag*PAI$^-$ isolates. We selected a group of 75 *H. pylori* isolates, 62 of which were *cag*PAI$^+$, 4 of which had a partial *cag*PAI (*cagA* negative), and 9 of which were *cag*PAI$^-$, to analyze their ability to induce IL-8 secretion (Fig. 2A). Among the *cag*PAI$^+$ *H. pylori* isolates tested, wide diversity in the ability to induce IL-8 secretion was observed, with IL-8 levels which ranged from <50 to over 1,500 pg/ml. IL-8 levels induced by *cag*PAI$^-$ isolates and by isolates with a partial *cag*PAI (*cagA* negative) were below 200 pg/ml in all cases, which is significantly lower than levels induced by *cag*PAI$^+$ isolates (Fig. 2A).

Isolates from individual patients, on the whole, gave similar results (Fig. 3). However, wide variation was seen between patients. For further analysis, we developed a definition of a non-IL-8-inducing isolate; using a cutoff value of the mean plus 3 standard deviations of the values obtained with the Tx30a reference strain and the nine *cag*PAI$^-$ isolates (<200 pg/ml), 16 of the 62 *cag*PAI$^+$ isolates were defined as negative for IL-8 induction (25.8%). Among these 16 isolates, 5 isolates were from patient 646 (6 isolates), 3 were from patient 248 (4 isolates), and a fraction of isolates were from patients 648, 555, 259, 256, 249, and 261 (Fig. 3).

Diversity in the induction of cellular elongation in AGS cells cocultured with *cag*PAI$^+$ and *cag*PAI$^-$ isolates. AGS cells were infected with the same 75 *H. pylori* isolates that were tested for IL-8 induction. Among the *H. pylori* *cag*PAI$^+$ isolates tested,

TABLE 2. Characteristics of *H. pylori* isolates tested for the presence of the *cag*PAI, the size of the *cagA* 3′ region, and activity on cells

Patient group	Patient	Diagnosis[a]	No. of isolates	*cag*PAI status[b]	Size of *cagA* 3′ region (bp)	No. of isolates used in AGS coculture expts/ no. of isolates sequenced	EPIYA pattern[c]
Children	365	CAP	20	Partial		4/0	
	646	CAP	20	+	500	6/2	ACC
	525	CAP	20	+	550	3/2	ABC
	648	CAP	20	+	550	5/2	AB&C
	236	CAP	20	+	570	4/2	ABC
	291	CAP	20	+	570	5/1	ABC
	482	DU	20	+	570	3/2	ABC
	475	CAP	20	+	650	9/2	ABCC
	307	CAP	20	+	650	4/2	AB&CC
	555	CAP	19	+	550	3/2	ABC
			1	+	650	1/1	ABCC
Adults	251	DU	14	−	−	3/0	−
	252	GU	14	−		2/0	
	254	NUD	17	−		2/0	
	248	GU	15	+	550	4/2	AB*C
	249	DU	15	+	800	2/2	ABCCC
	256	DU	16	+	850	4/2	ABABC
	259	DU	8	+	550	5/2	ABC
			2	+	500	2/2	ABC
			3	−		2/0	
	261	GU	13	+	550	1/1	AB&C
			1	+	800	1/1	AB&AB&C
	Total		318			75/30	

[a] CAP, chronic abdominal pain; DU, duodenal ulcer; GU, gastric ulcer; NUD, nonulcer dyspepsia.
[b] +, present; −, absent.
[c] B* motif, EPTYAQVAKKV; B& motif, EPIYTQVAKKV.

there was wide diversity in the ability to induce cellular elongation, with values ranging from <10% to over 70% of cells affected. *cag*PAI$^-$ isolates and isolates with a partial *cag*PAI (*cagA* negative) induced elongation in <20% of the cells (Fig. 2B); these values were significantly lower than those induced by *cag*PAI$^+$ isolates. In general, isolates from the same patient induced similar values, although wide variation was seen between patients. Most strains inducing high levels of IL-8 also caused the most cytoskeletal changes, although there were clear exceptions (e.g., patients 307, 475, 249, and 261)(Fig. 3). We defined the cutoff value for cellular elongation as the mean plus 3 standard deviations of the values obtained with the Tx30a reference strain (negative control) and nine *cag*PAI$^-$ isolates (<20%). Using this cutoff value, 11 of the 62 (17.7%) *cag*PAI$^+$ isolates were negative for cellular elongation, with 4 of 4 isolates from patient 248 and a fraction of isolates from patients 648, 646, and 555 being negative (Fig. 3).

***H. pylori* adherence and viability during coinfection.** *cag*PAI$^+$ isolates with low or high activity on AGS cells were monitored for adherence and viability. Both types of isolates, either positive or negative for cell activity, showed similar patterns of adherence to AGS cells, which increased over time, as shown in Fig. S1 in the supplemental material; the mobility of bacteria was evident during the 48 h of the assay. Viable *H. pylori* cells (>10^{10} CFU/ml) could be recovered on agar plates even after 48 h of coinfection with both types of isolates; in addition, images show the growth of *H. pylori* colonies on the surfaces of AGS cells after hours of coinfection (see Fig. S1 in the supplemental material). In summary, no difference was observed in adherence, mobility, or viability between isolates with low or high activity on cells.

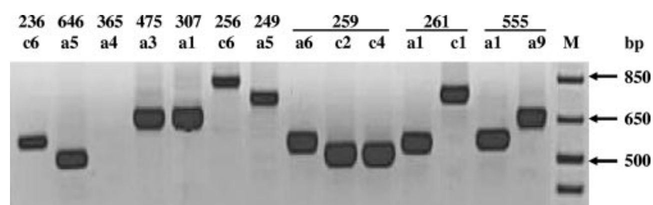

FIG. 1. Determination of the size of the *cagA* 3′-variable region by PCR. PCR products from single-colony isolates from different patients or from the same patient (259, 261, and 555) showed size variation. Lane M, molecular size marker.

Translocation and phosphorylation of CagA in AGS cells. The above results showed that *H. pylori cag*PAI$^+$ isolates had variable effects on AGS cells. To further study this variability, we analyzed the translocation and phosphorylation of the CagA protein in the same 75 isolates in AGS cells. We observed differences in the size of the CagA proteins, which correlated with variation in the size of the 3′-variable region of *cagA* (Fig. 4). According to our results, 58 of the 62 *cag*PAI$^+$ isolates tested had CagA translocated and phosphorylated within AGS cells. In the remaining four isolates (all from patient 236), the CagA protein was not recognized by the commercial polyclonal anti-CagA antibody (bN-20) we used; however, the translocated CagA protein was recognized by the anti-phosphorylated tyrosine antibody (PY99) (Fig. 4). In addition, the CagA proteins from these isolates were recognized by immunoglobulin G in sera from two *H. pylori*-infected patients (see Fig. S2 in the supplemental material).

As expected, the isolates with a partial *cag*PAI, with absence of the *cagA* gene, and the *cag*PAI$^-$ isolates did not express the

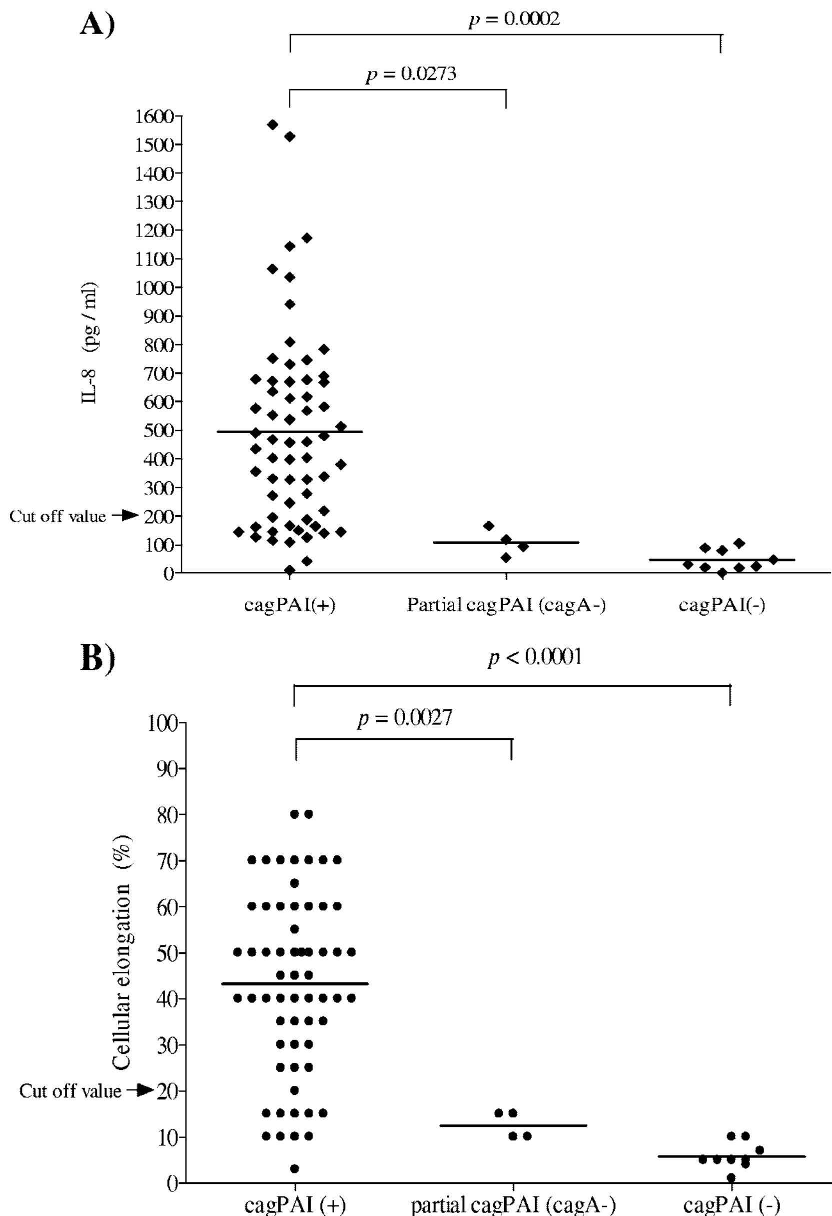

FIG. 2. Diversity in biological activities on AGS cells of 62 *cag*PAI$^+$ isolates, 4 isolates with a partial *cag*PAI (*cagA* negative), and 9 *cag*PAI$^-$ isolates. (A) Induction of IL-8 expression. (B) Induction of cellular elongation. The intensities of both activities varied considerably among the *cag*PAI$^+$ isolates and were significantly higher than those of partial *cag*PAI and *cag*PAI$^-$ isolates. Horizontal lines indicate mean values. The results represent the means for three independent experiments. The cutoff values for both activities were estimated as the means plus 3 standard deviations of the values obtained with the Tx30a reference strain and nine *cag*PAI$^-$ isolates.

CagA protein and did not have phosphorylated CagA in AGS cells. Of interest, the *cag*PAI$^+$ isolates that were noninducers of either cellular elongation or IL-8 secretion were still able to translocate and phosphorylate CagA in AGS cells, although in several cases (such as isolate 648a9) phosphorylation was weak (Fig. 4).

Diversity in the biological activities of multiple *H. pylori* isolates from single patients. Heterogeneity in the activities of multiple isolates from single patients on AGS cells was analyzed. Although most isolates from single patients were rather homogeneous with regard to cell activity (Fig. 3), significant diversity was observed in some cases. Isolates from patients 555, 256, and 259 displayed a high level of diversity in both cellular elongation and IL-8 induction. In addition, we identified isolates causing a strong cellular elongation phenotype which were negative for IL-8 induction (from patients 555, 646, 249, 256, 259, and 261). Isolates negative for cellular elongation but positive for IL-8 induction (from patients 555, 648, and 248) were also found, although in all these cases induction of IL-8 was rather weak (Fig. 3).

Sequencing of the 3′-variable region of the *cagA* gene. From the 62 *cag*PAI$^+$ *H. pylori* isolates tested in the above studies, we chose 30 isolates for sequencing, representing the different sizes in the 3′ region of the *cagA* gene and different levels of

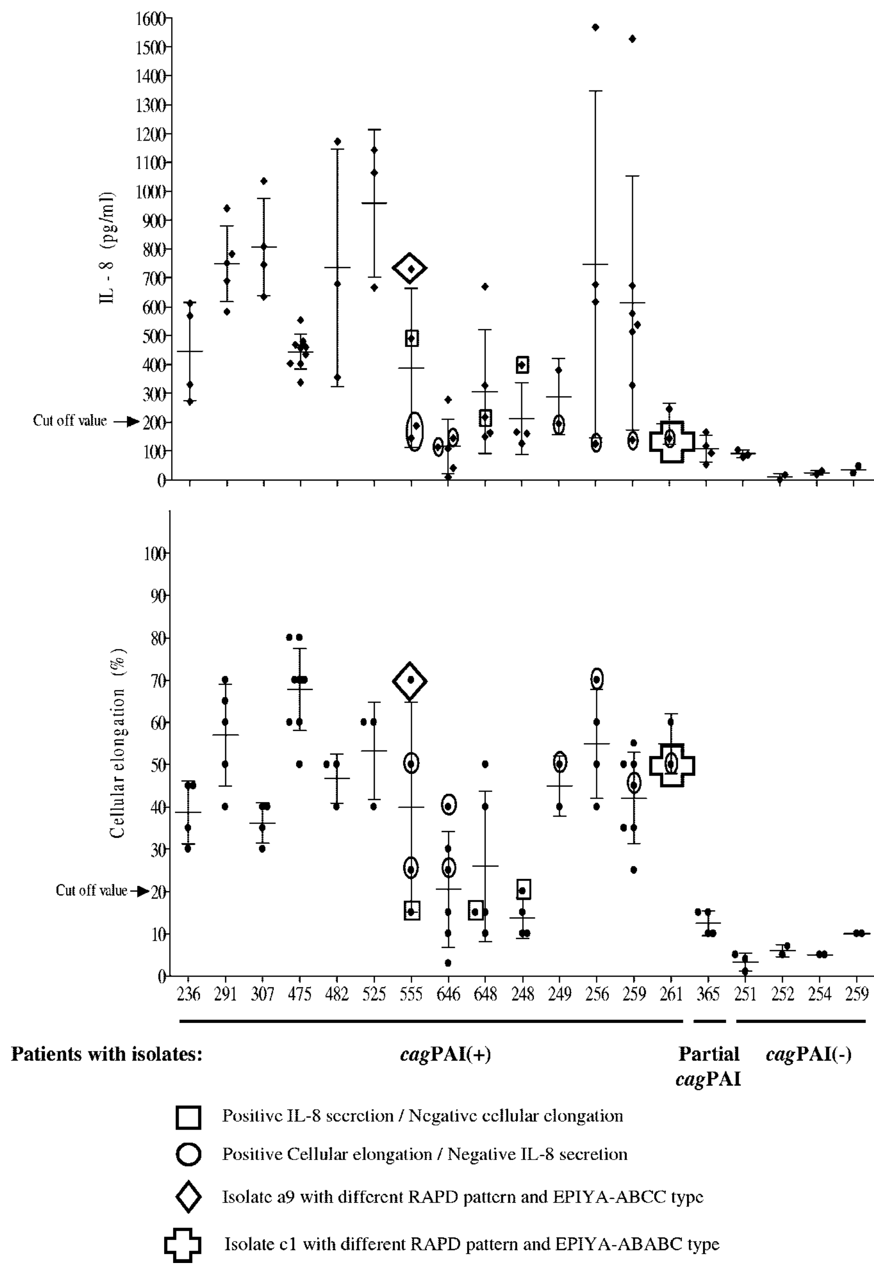

FIG. 3. Diversity in the induction of cellular elongation and IL-8 secretion by multiple *cag*PAI$^+$ *H. pylori* strains, isolates with a partial *cag*PAI, and *cag*PAI$^-$ isolates from single patients. Patient numbers are indicated on the *x* axis, and intensities of cell activities are shown on the *y* axes. Horizontal lines indicate mean values, and bars indicate standard deviations. The results represent the means for three independent experiments.

activity on AGS cells (Table 2). For all 30 of these isolates, *cagA* had the Western type sequence (Fig. 5). The number of EPIYA motifs varied among these isolates; 20 isolates (66.6%) had three motifs, 5 (16.7%) had four motifs, and 5 (16.7%) had five motifs. The majority of the isolates with three EPIYA motifs were of the ABC type, and only two isolates (from patient 646) were of the ACC type, with a deletion between the A and C motifs. All 20 of these isolates had a 3′ *cagA* size of ≤570 bp (Table 2). The isolates with four EPIYA motifs were of the ABCC type, and all had a 3′ *cagA* size of 650 bp. Among the five isolates with five EPIYA motifs, two were of the ABCCC type and three were of the ABABC type, and they had 3′ *cagA* sizes of between 800 and 850 bp (Table 2).

Isolates from patient 259 yielded 3′ *cagA* PCR products of 500 bp (isolates c2 and c4) and 550 bp (isolates a1 and a6); these PCR products were sequenced. Compared with isolates a1 and a6, isolate c2 had a deletion of ~24 amino acids after the EPIYA-C motif (Fig. 5); of interest, another isolate from the corpus, c4, also showed a deletion after the EPIYA-C motif, although this was shorter (~15 amino acids). All isolates from this patient showed similar RAPD patterns, suggesting that they were the same strain. However, isolate c2 was nega-

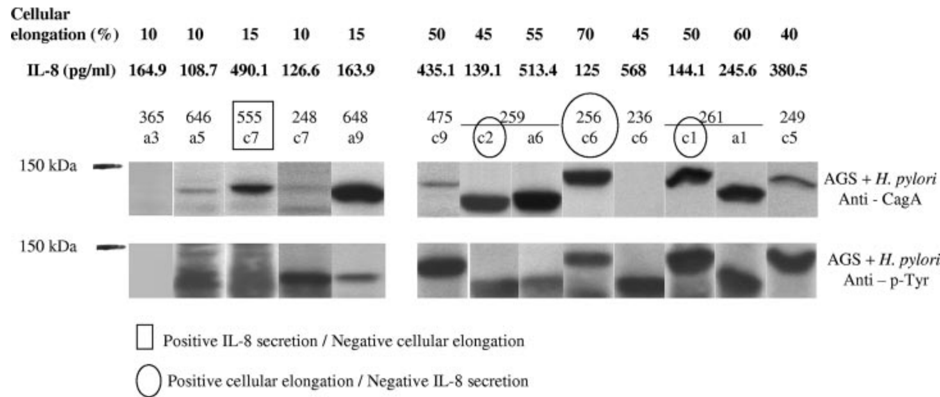

FIG. 4. Translocation and phosphorylation of the CagA protein in AGS cells infected with *cag*PAI^{+} and partial *cag*PAI-carrying *H. pylori* isolates. Western blot analysis of CagA translocation into AGS cells was performed with a polyclonal anti-CagA antibody, and detection of CagA phosphorylation was done with an anti-phosphotyrosine monoclonal antibody. Isolate 365a3 is a partial *cag*PAI-carrying (*cagA* negative) isolate; all other isolates (from 646a5 to 249c5) are *cag*PAI^{+}. Isolate 236c6 was negative for Western blotting with anti-CagA but positive with anti-phospho-Tyr. Isolates with variability in biological activity levels are marked with circles or squares.

tive for IL-8 induction, while isolates a1, a6, and c4 were positive, suggesting that the long deletion after the EPIYA-C motif might be associated with a loss of biological activity. Among the 20 isolates tested from patient 555, 19 had a 3′ *cagA* size of 550 bp and an ABC pattern, whereas one had a size of 650 bp (a9) and an ABCC pattern. Isolate a9 displayed higher levels of IL-8 secretion and cellular elongation than did isolates with the ABC pattern; isolate a9 had a different RAPD pattern, implying that it was a different coinfecting strain. Fourteen isolates were tested from patient 261; 13 had a 3′ *cagA* size of 550 bp and an ABC pattern, whereas one had a size of 800 bp (c1) and an ABABC pattern. IL-8 secretion and cellular elongation levels were lower in the isolate with the ABABC pattern. Isolate c1 also had a different RAPD pattern (data not shown), implying that it was a different coinfecting strain (Fig. 3). Thus, of three patients with isolates differing in the size of the 3′ *cagA* region, two had mixed infections and one had the same strain with polymorphism in the 3′ sequence.

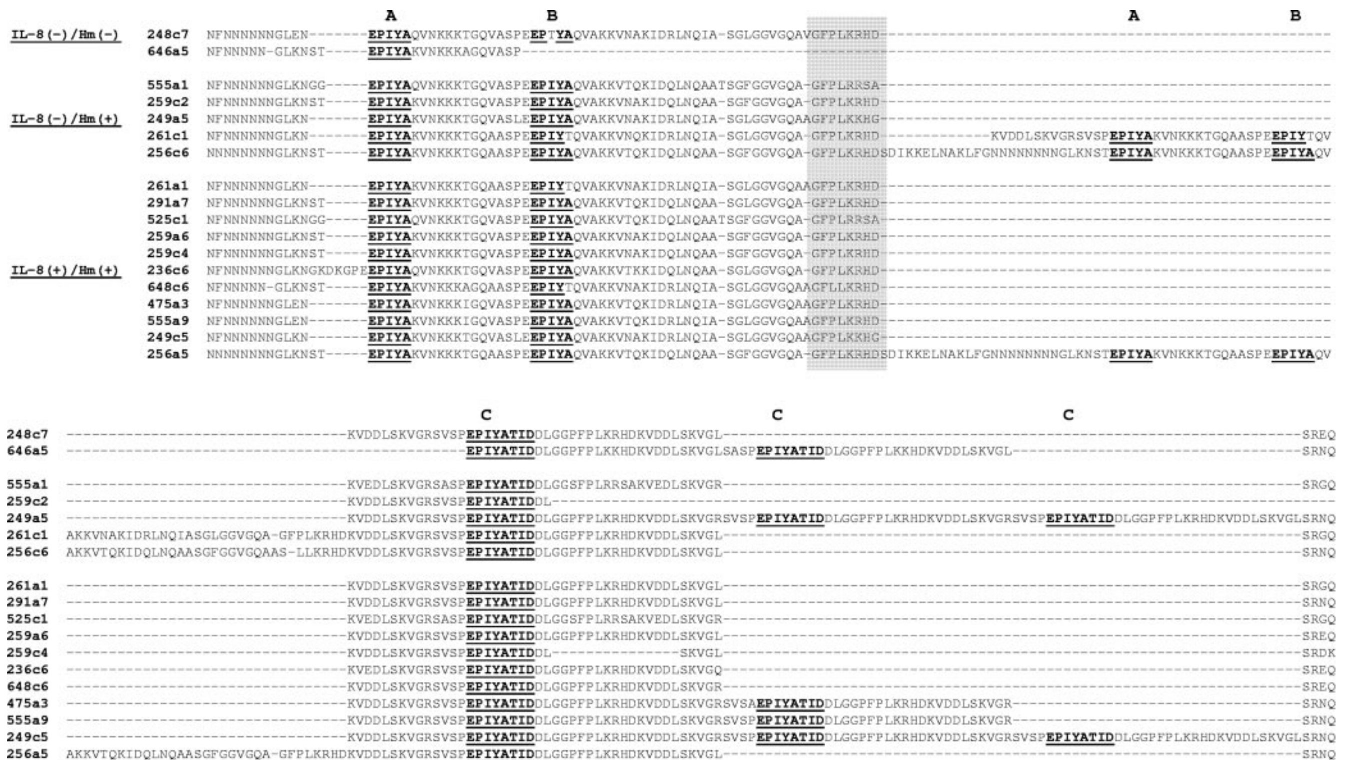

FIG. 5. Deduced amino acid sequences of C-terminal repeat regions of CagA from *cag*PAI^{+} isolates that were positive or negative for induction of IL-8 or cellular elongation (Hm). The EPIYA-A, -B, and -C motifs and the SHP-2 binding site after the EPIYA-C motif are underlined, and the Western sequence type is shaded.

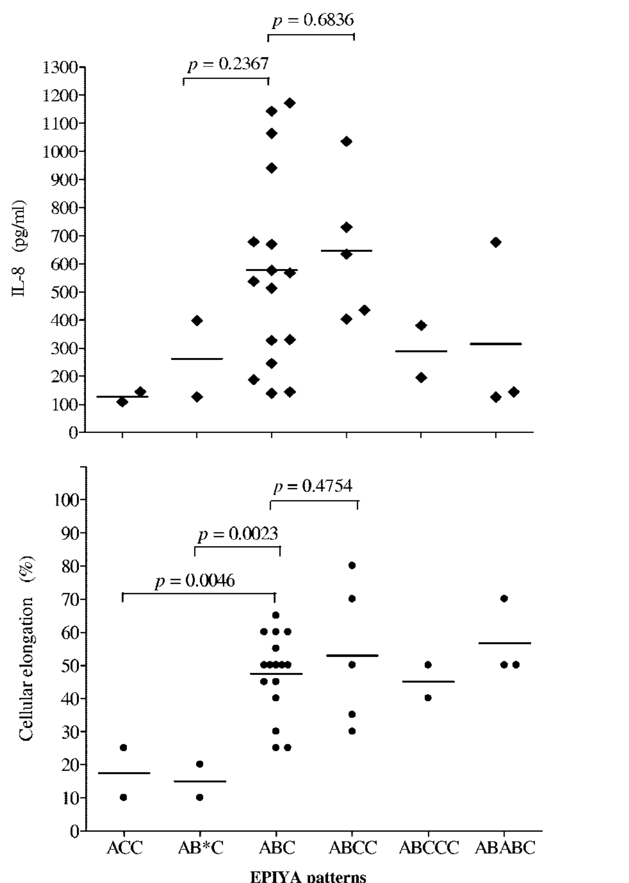

FIG. 6. Relationship between EPIYA patterns and the ability to induce both IL-8 secretion and cellular elongation for 30 *cag*PAI$^+$ *H. pylori* isolates from our population. Isolates without the B motif (ACC pattern; patient 646) or with a modified B motif (EPTYA; patient 248) had a significantly weaker cellular elongation phenotype.

Association of the sequence in the 3′ region of *cagA* with activity on AGS cells. We next analyzed the sequence of the 3′-variable region, searching for a possible association with activity on cells, especially for isolates which lacked activity on AGS cells. There was no clear association between the number and type of EPIYA motifs and the ability to induce IL-8 secretion or cellular elongation (Fig. 6). However, several interesting results were found in the sequences for isolates negative for both cellular elongation and IL-8 induction; some had an EPIYA-ACC pattern (patient 646) (Fig. 5), suggesting the importance of the EPIYA-B motif for cell activity. Isolates from patient 248 with an EPIYA-ABC pattern had a modified B motif (B* [EPTYA]); three of four isolates were negative for IL-8 induction, and all four were negative for induction of cellular elongation (Fig. 6). Isolates from patient 307 with an EPIYA-ABCC pattern had a different modification of the B motif (B$^{\&}$ [EPIYT]) and induced lower levels of cellular elongation and IL-8 secretion than did isolates with the normal ABCC pattern (Fig. 6).

For isolates from patient 236 that were *cagA* positive but whose protein products were not recognized by the commercial anti-CagA polyclonal antibody (Fig. 1), we observed a short insertion of five amino acids (DKGPE) before the EPIYA-A motif which was not observed for isolates from any other patient (Fig. 5); of interest, isolates from this patient caused moderate IL-8 and cellular elongation induction (Fig. 3).

DISCUSSION

cagA is a highly polymorphic gene with diversity in its 3′-end region, which is important for the biological activity of the encoded protein on gastric epithelial cells. This region encodes the tyrosine phosphorylation site and the SHP-2 phosphatase binding site. The interaction of these proteins activates a series of signaling pathways, causing an increase in proliferation and abnormal cell motility (45, 46). It is therefore important to extend studies on polymorphism in this 3′ region of *cagA* across populations and to correlate this diversity with the biological activities of strains. In the present study, we addressed both the extent of polymorphism in the *cagA* gene and the diversity in the biological activities of Mexican strains on gastric epithelial cells.

To better describe the extent of polymorphisms, we chose to study multiple single colonies isolated from different sites of the stomach of individual patients. All isolates from the antra and corpora of 13 of the 18 patients studied had a homogeneous *cag*PAI content, one had a partial *cag*PAI content (patient 365), and for only one patient was a mixed infection with *cag*PAI$^+$ and *cag*PAI$^-$ strains documented (patient 259). However, in spite of the rather homogeneous gene content in the *cag*PAI, diversity in the sequence of the *cagA* 3′ region was observed in isolates from three patients. In one case, the multiple isolates were found to be of the same strain (patient 259), and in two cases, the strains were not the same as those documented by RAPD analysis (patients 555 and 261); thus, mixed infection was documented in two cases and polymorphism in the 3′ region was shown for one patient.

Once we had determined that most isolates in this study were positive for the *cag*PAI, we next analyzed phenotypic diversity by studying the heterogeneity in the activities of these isolates on epithelial cells. *cag*PAI$^-$ isolates and isolates with a partial *cag*PAI (*cagA* negative) induced very low levels of cellular elongation ($<$20%) and IL-8 ($<$200 pg/ml), as expected. On the other hand, even among isolates positive for the four genes of *cag*PAI tested, including *cagA*, there were a number of isolates which were unable to induce IL-8 secretion or cellular elongation, which suggests that the mere presence of the *cag*PAI is not sufficient for these activities. In several of these cases, low phosphorylation of CagA might explain the low cell activity, but in others negative activity was observed in spite of the translocation and phosphorylation of CagA. It should be stressed that in all cases, the CagA protein was translocated and phosphorylated, suggesting the presence of a functional type IV secretion system. Polymorphisms in the 3′ *cagA* region explained many of these cases and illustrate the importance of the EPIYA-B motif; thus, strains lacking this motif (ACC pattern) were unable to induce either IL-8 secretion or cellular elongation, an observation which has not been reported previously. In addition, strains with a modified B motif (EPTYA) also displayed weak activity, which further confirms the importance of the whole motif for cell activity. Deletions after the EPIYA-C motif were also associated with either a low or

absent biological activity. These results show that negative or weak IL-8 or cellular elongation induction in *cag*PAI$^+$ isolates might be due to specific modifications in the CagA sequence. In accordance with these results, Brandt et al. recently reported that among *cag*PAI$^+$ strains there are high and low IL-8 inducers, and this variability was associated with the number of EPIYA motifs and the amino acid residues surrounding these motifs, stressing the need for CagA to induce IL-8 in epithelial cells (14). The diversity in the activities of different *cag*PAI$^+$ *H. pylori* isolates on AGS cells does not appear to be influenced by the degree of adherence of the isolates to AGS cells or their ability to grow during the coinfection experiment, since we found similar adherence and growth on AGS cells by *H. pylori* isolates with either high or low cell activity.

Of note, among the *cag*PAI$^+$ isolates, the range of IL-8 induction varied as much as 30 times (from 50 to over 1,500 pg/ml), and the intensity of induction of cellular elongation varied over 15 times (from 5 to 80%); such wide diversity has not been documented previously, and as discussed below, polymorphisms in the *cagA* gene partially explain this diversity. Higashi et al. described that the variation in biological effects of CagA is caused by the variation in the number and sequence of tyrosine phosphorylation sites (21). This correlation was not observed in our study, as exemplified by the fact that IL-8 and cellular elongation activities of isolates with an ABC pattern varied from no to high induction. The amount of CagA translocated and phosphorylated had a correlation with low activity in several isolates but not in others. As discussed above, in some of these cases variations in the sequence of the B motif might explain the observed low activity on cells. Still, when cellular elongation was analyzed, the degree of induction tended to increase in the direction ACC→AB*C→ABC→ABCC→ABABC, which partially agrees with previous reports (4, 31). This correlation was not observed for IL-8 induction, which is also in agreement with previous reports (3, 4, 35) and confirms that although the role of CagA in both activities is controversial, the mechanism of action is different for each activity (41). This is further supported by the fact that isolates from six patients displayed a moderate or strong cellular elongation activity but no IL-8 induction. Some reports have proposed a key role for *cagE* in IL-8 induction (35); however, our results disagree with this, as all isolates with negative or low IL-8 induction had the *cagE* gene present. Still, we cannot discard the possibility that like the case for *cagA*, sequence diversity in *cagE* might cause differences in IL-8 induction. Other authors have also reported that *H. pylori* can induce NF-κB activation (and IL-8 secretion), via Nod1, in epithelial cells that respond to peptidoglycan delivered to the cytoplasm of the cell by the type IV secretion system encoded by the *cag*PAI (49).

When phenotypic diversity was analyzed in multiple isolates from a single patient, with all isolates representing unique strains, in many cases a rather homogeneous degree of activity was documented among the isolates. However, there were patients for whom the activities of the isolates on cells varied over 10 times. Some of these cases might be explained by polymorphisms in the *cagA* sequence among the colonizing isolates; sequence diversity, insertions, or deletions were documented in such cases. However, in other cases, such diversity cannot be explained by heterogeneity in the *cagA* sequence, and polymorphisms in other genes of the *cag*PAI or outside the island might help to explain this diversity. It should be noted that isolates of a single patient classified as the same isolate with tests such as RAPD or amplified fragment length polymorphism analysis may still show variation in the gene content of 3 to 5% of the genome (12, 23), and this variation may cause phenotypic diversity. The mechanisms responsible for the observed diversity in these patients deserve further study.

An interesting case was patient 236, whose *H. pylori* strain produced a CagA protein which could not be recognized by the commercial antibody used in this study; sequence studies showed that it had a five-amino-acid insertion before the EPIYA-A motif. Conceivably, this might cause changes in the structure of the protein sufficient to avoid recognition by particular antibodies. However, this modified protein was still recognized by serum from the same patient from whom the strain was isolated as well as by sera from other patients.

In conclusion, our study documents a high level of diversity in the ability of *cag*PAI$^+$ strains to induce both IL-8 production and cellular elongation in epithelial cells; such diversity might be explained partially by polymorphisms in the 3′ region of *cagA*. Another novel observation was the finding that isolates lacking the EPIYA-B motif (pattern ACC) or with a modified B motif displayed a negative or weak activity on epithelial cells; to our knowledge, such a possible association has not been reported previously. We documented important diversity in the activities of isolates from single patients on cells, which in some, but not all, cases could potentially be explained by polymorphisms in *cagA*.

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