INFECTION AND IMMUNITY, Mar. 2001, p. 1625–1629 Vol. 69, No. 3
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.3.1625–1629.2001
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Implication of the Structure of the *Helicobacter pylori cag* Pathogenicity Island in Induction of Interleukin-8 Secretion

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Received 12 September 2000/Returned for modification 19 October 2000/Accepted 4 December 2000

***Helicobacter pylori* virulence is associated with the presence of the *cag* pathogenicity island (PAI). The *cag* PAI is involved in the ability to induce interleukin-8 (IL-8) secretion by human cells, which is implicated in the inflammatory response of the gastric mucosa to *H. pylori* infection. The aim of this study was to determine whether the genetic structure of the *cag* PAI is conserved and whether it is linked to IL-8 induction ability. Detection of specific markers (*cagA, picB, cag13-cag14, virD4*, and IS*605*) by PCR and dot blot hybridization and long-distance PCR determination of the presence of cagI, cagII, and the middle region of the *cag* PAI were performed on 153 strains isolated from adults suffering from ulcers ($n = 79$) or gastritis ($n = 74$). IL-8 induction ability was evaluated by coculture of the strains with HEp-2 cells. Eighty-three strains (54.3%) had an entire *cag* PAI, 12 strains (7.8%) had the *cag* PAI split in two, 49 strains (32%) had no *cag* PAI, and 9 strains exhibited other structural combinations. The presence of an entire *cag* PAI was statistically correlated with the presence of IS*605* ($P = 0.006$) and the ability to induce IL-8 secretion but not with clinical presentation of the infection. The structure of the *cag* PAI appears to be rather conserved and is related to the proinflammatory power of a strain. The existence of strains inducing IL-8 secretion regardless of the *cag* PAI structure suggests that this region is not the only requirement for IL-8 secretion.**

Helicobacter pylori is recognized as the causative agent of gastritis and ulcer disease and is also a major risk factor for gastric cancer or mucosa-associated lymphoid tissue lymphoma (5, 12, 13, 21). The pathogenesis of the diseases associated with *H. pylori* is not yet fully understood, although several hypotheses have been proposed (3). The polymorphism of the clinical manifestations associated with *H. pylori* infection is potentially due to differences in virulence among individual *H. pylori* strains (3, 6, 7, 16, 29). Nevertheless, there are no reliable virulence markers that can be used to predict the severity of the disease associated with *H. pylori* infection.

One of the most studied putative virulence factors is the CagA protein, encoded by the *cagA* gene (9, 27). This gene is reported to be present in 60 to 70% of European isolates (9, 27), and the presence of *cagA* is statistically associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer (6, 9, 29). The *cagA* gene is one of the 31 genes of a pathogenicity island (PAI) called the *cag* PAI (7). The presence of *cagA* is considered a marker of presence of the *cag* PAI (1, 7).

The *cag* PAI is a 40-kb locus inserted in the chromosomal glutamate racemase gene (7). Its G+C content (35%) differs from the G+C content of the rest of the genome (39%), suggesting that it was acquired from another organism by horizontal transfer (1, 2, 7, 26). Several studies have revealed that the *cag* PAI can be found either as a single uninterrupted unit, split into two regions (cagI and cagII) either by an insertion sequence called IS*605* or by a large piece of chromosomal DNA, or partially deleted (7, 9, 15, 20).

It has been shown that the *cag* PAI is involved in the induction of interleukin-8 (IL-8) secretion by human cells colonized by *H. pylori*. This property may be related to the proinflammatory power of a strain and thus to its virulence. Indeed, strains inducing IL-8 secretion have been associated with the presence of the *cagA* gene and with severe diseases (11, 23), although this has been contested recently (15, 20). Knockout of most of the genes of this region (except *cagA* and *cagN*) resulted in a decrease or a suppression of the IL-8 induction ability of a strain (1, 7, 10, 23, 28). The mechanism of IL-8 induction is not yet understood. However, some genes of the *cag* PAI are homologous to genes of a type IV secretion pathway, suggesting that this region encodes a secretion system involved in the export of virulence determinants (7, 8, 28).

Because the *cag* PAI has a variable genetic structure and because this structure may influence the IL-8 induction ability of the strain, we aimed to determine the *cag* PAI structure for a substantial number of French strains in order to learn whether the structure of this region is conserved and whether certain *cag* PAI structures are specifically associated with the ability to induce IL-8 secretion.

MATERIALS AND METHODS

Patients. Biopsy specimens were sampled from 153 consecutive *H. pylori*-infected adults from a group of patients who had undergone upper gastrointestinal endoscopy in the Gastroenterology Department of Poitiers University Hospital (Poitiers, France) because of dyspeptic complaints. None of the patients was receiving antisecretory or nonsteroidal anti-inflammatory drugs. The patient population consisted of 107 males (aged 17 to 95 years; mean age, 48.2 years), and 46 females (aged 22 to 86 years; mean age, 57.6 years). The presence of an ulcer was based on endoscopic examination of the stomach and duodenum. Each patient's history was investigated for an earlier diagnosis of peptic ulcer. If the patient's documented history or endoscopic examination revealed peptic ulcer disease, the patient was considered to have an ulcer. Otherwise, he or she was considered to have dyspepsia or gastritis only. Patients were assigned to four groups: duodenal ulcer ($n = 47$), gastric ulcer ($n = 22$), both duodenal and gastric ulcers ($n = 10$), and nonulcer dyspepsia or chronic gastritis ($n = 74$).

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TABLE 1. Primers used to study the *cag* PAI structure

Amplified area (reference)	Primer names	Sequence (5′→3′)
cagA (27)	F1	GATAACAGCCAAGCTTTTGAGG
	B1	CTGCAAAAGATTGTTTGGCAGA
picB	picB/AF	GGCTTTATCAAAGAATGGAGCGAGCG
	picB/AR	TACTCAATAGCTCTTCTATGAGC
	picB/BF	GTGGAGGCTCTAAAGAGCAGGGC
	picB/BR	TGACATACTCCCCACCCATTGCG
cag13-cag14	cag13F	CAATAGTGGGAGCTTAGTGCC
	cag14R	GCGATTGGTGGCTACTTATCGC
virD4	virD4AF	TTTATGATGATAATCGATCGCC
	virD4AR	GAACGCCTGCCCTGCGTAAGCG
	virD4BF	TTTCATAGGTTCTATGGCAAGCGGG
	virD4BR	TTAGCGCCATTCCTACCATAACC
cag empty site (19)	Luni1	ACATTTTGGCTAAATAAACGCTG
	5280	GGTTGCACGCATTTTCCCTTAATC
IS*605* TnpA	605AF	CGCCTTGATCGTTTCAGGATTAGC
	605AR	CAACCAACCGAAGCAAGCATAATC
IS*605* TnpB	605BF	GGCTGTTCTAGGGTCGTGTATAAC
	605BR	CAAGCTAGATGATGCAATCTAGCTACC
Full IS*605* (18)	ORF18F	CGCCTTGATCGTTTCAGGATTAGC
	ORF19R	CAAGCTAGATGCAATCTAGCTACC
cagII	cag1F	TGTTGTGTCTTGAGCGGTGC
	cag9R	GGCGATGGTCTCTTTTATCGCC
Middle zone	cag9F	GGCGATAAAAGAGACCATCGCC
	cag18R2	GCTGAGCAATGCGGAATAT
cagI	cag18F2	ATATTCCGCATTGTTGCTCAGC
	cag26R	GCTTGCCTGTTATCCCTATCG

***H. pylori* strains and culture conditions.** Gastroduodenal biopsy specimens were sent to the laboratory in 1 ml of transport medium. The specimens were ground, spread on Columbia agar plates containing 5% horse blood (Bio-Merieux, Marcy l'Etoile, France), and incubated under microaerobic conditions at 37°C for 2 to 5 days. Bacteria were identified as *H. pylori* by standard criteria, and the strains were stored at −80°C in 10% glycerol broth. These were referred to as stock cultures. Three reference strains were used as controls: strains ATCC 26695, J99, and Tx30a.

DNA isolation. DNA was isolated from the cultured bacteria by harvesting cells from a plate and resuspending them in 500 μl of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). Bacteria were lysed by addition of 50 μl of a 10% sodium dodecyl sulfate solution and 1.25 μl of a 20-mg/ml proteinase K solution (Merck, Darmstadt, Germany). The mixture was incubated for 1 h at 37°C. Proteins were precipitated by 500 μl of phenol-chloroform-isoamyl alcohol (Eurobio, Les Ullis, France). After centrifugation, the supernatant was removed and DNA was precipitated twice with absolute ethanol at −20°C and washed with 75% ethanol at −20°C (Merck). After centrifugation, the pellet was dried and resuspended in 200 μl of deionized water containing 50 μg of RNase (Merck)/ml. The mixture was incubated for 1 h at 60°C, and the DNA extracts were stored at −20°C.

PCR-based typing and long-distance PCR. PCRs were performed in a volume of 100 μl using r*Taq* polymerase (Pharmacia Biotech, Orsay, France). The conditions used were those recommended by the manufacturer. The primers used to detect the presence of *cagA, picB, virD4, cag13-cag14*, IS*605*, and the *cag*-empty site are listed in Table 1. PCR amplification was performed under the usual conditions. For the long-distance PCR investigation, the primers used (Table 1) were designed so that the amplified products covered the entire *cag* PAI: from *cag1* to *cag9*, from *cag9* to *cag18*, and from *cag18* to *cag26* (see Fig. 1). The long-distance PCR amplification was performed with the Long-PCR kit (Perkin-Elmer), as recommended by the supplier. PCR products were visualized by electrophoresis on 1.8% agarose gels (Eurobio) after 20 to 30 min of migration at 150 V.

Determination of *cagA, picB, virD4*, and *cag13-cag14* status by dot blot hybridization. To test the sensitivity and the specificity of PCR with primers specific for *cagA, picB, virD4*, and *cag13-cag14*, the 153 strains were analyzed by dot blotting using a method previously described (4). Briefly, *cagA, picB, virD4*, and *cag13-cag14* probes were generated by PCR from chromosomal DNA of *H. pylori* ATCC 26695 using primers F1 and B1 for *cagA*, primers 544BF and 544BR for *picB*, primers 524AF and 524AR for *virD4*, and primers cag13F and cag14R for *cag13-cag14*. DNA from the 153 *H. pylori* strains was filtered through a 0.45-μm-pore-size nitrocellulose membrane (Bio-Rad, Ivry sur Sein, France) using a 96-well dot blot apparatus filtration system. DNA probes, labeled by random priming, were incubated with the membrane, and autoradiography was performed with X-ray film (Kodak).

IL-8 induction by *H. pylori* strains. The IL-8 induction ability of *H. pylori* was investigated on HEp-2 cells as previously described (4). Briefly, we cocultured HEp-2 cells for 24 h with an *H. pylori* suspension containing 5×10^8 bacteria/ml. Negative and positive controls were included. The medium was removed, and the IL-8 produced in the supernatant was evaluated by an enzyme-linked immunosorbent assay (ELISA) using the specific ELISA kit provided by Diaclone (Besançon, France) according to the manufacturer's instructions.

Statistical method. Analysis of data was performed by using the chi-square test with Yates' correction. Probability levels (P) of <0.05% were considered statistically significant.

RESULTS

Statuses of the strains with regard to various genes of the *cag* PAI. The presence of the *cagA, picB, virD4*, and *cag13-cag14* genes and of IS*605* was determined by PCR and dot blot hybridization. Those genes were choosen because they are well distributed over the *cag* PAI. Strains were considered negative for a gene if they tested negative by both PCR and dot blot hybridization. All the other strains were considered positive. The correlation between results obtained for the various genes by PCR and by dot blot hybridization ranged from 95 to 100%. The statuses of the 153 strains with regard to the *cagA, picB, virD4*, and *cag13-cag14* genes and IS*605* are shown in Table 2. None of these five genes correlated with the clinical manifestations of the infection.

The presence of the *cag* PAI (*cag* PAI status) was systematically investigated by PCR using primers directed to the regions flanking the *cag* PAI. Therefore, if a full or partial *cag*

TABLE 2. Statuses of 153 strains with regard to various genes and fragments of the *cag* PAI

Structure of *cag* PAI	No. (%) of strains	No. of strains from patients with:		No. of strains with the indicated gene[b]					No. of strains with the indicated region[c]		
		NUD[a]	Ulcer	*virD4*	*cag13–cag14*	*picB*	*cagA*	IS*605*	cagII	Middle	cagI
Uninterrupted	83 (54.3)	40	43	81	70	82	82	22	64	78	53
No *cag* PAI	49 (32)	8	4	0	0	2	0	5	0	0	0
Split in two	12 (7.8)	23	26	12	0	12	11	6	9	0	8
Other[d]	9 (5.9)	3	6	1	3	3	6	0	1	4	1

[a] NUD, nonulcer dyspepsia.
[b] By PCR and dot blot hybridization.
[c] By long-distance PCR.
[d] One strain had the middle region and cagII, two strains had the middle region and cagI, one strain had cagII only, one strain had cagI only, one strain had the middle region only, and three strains had *cagA* only.

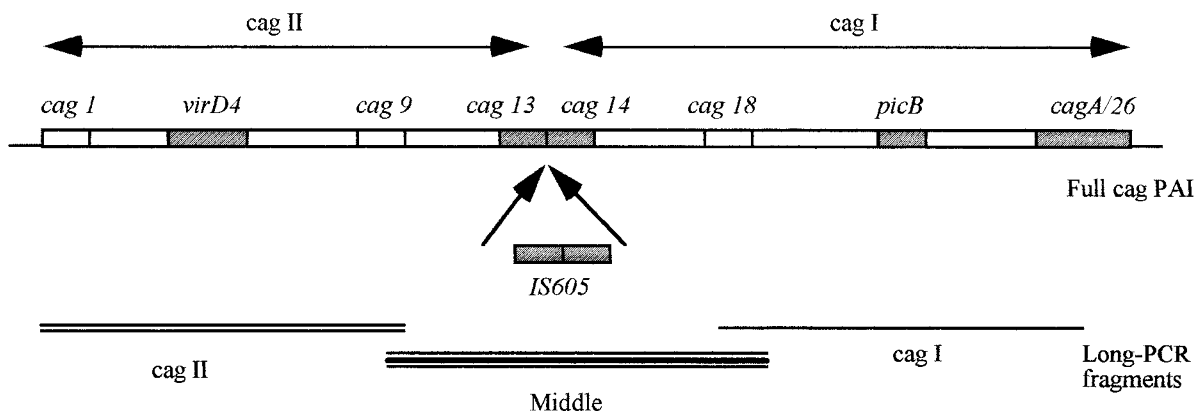

FIG. 1. Area amplified by long-distance PCR. Boxes, genes, detected by PCR and dot blot analysis.

PAI was present, the PCR could not be completed because of the size of the fragment to be amplified. If the *cag* PAI was absent, an amplification product of 500 bp was obtained. In this study we amplified the 500-bp fragment for 49 of 153 strains (32%), suggesting that these 49 strains did not have a *cag* PAI. Most, if not all, of these 49 strains were negative for the *cagA, picB, virD4*, and *cag13-cag14* genes and for IS*605* (49, 47, 49, 49, and 44 strains, respectively, were negative for the genes indicated) as determined by PCR and dot blot hybridization, confirming the absence of the *cag* PAI.

Structure of the *cag* PAI as determined by long-distance PCR. The structure of the *cag* PAI was investigated using long-distance PCR. Primers were selected so that the amplified products covered the whole *cag* PAI (Fig. 1). The presence of the cagI part of the *cag* PAI was determined using primers specific to the *cag18* and *cag26* genes, that of cagII was determined using primers specific to the *cag1* and *cag9* genes, and that of the middle region of the *cag* PAI, where the separation mediated by IS*605* is thought to occur, was detected using primers specific to the *cag9* and *cag18* genes. The prevalence of each of these fragments is shown in Table 2.

To establish the structure of the *cag* PAI, we hypothesized that the strains possessed the cagII part of the *cag* PAI when they were positive for *virD4* or by long-distance PCR with primers cag1F and cag9R. They were considered positive for the middle region of the *cag* PAI when they were positive for *cag13-cag14* or by long-distance PCR with primers cag9F and cag18R2. They were considered positive for the cagI part of the *cag* PAI when they possessed *cagA* and *picB*, or if they were positive by long-distance PCR with primers cag18F2 and cag26R. All the strains bearing at least one of these three fragments had positive *cag* PAI statuses (see above). Strains were considered negative for the portion studied when they were negative with by PCR, dot blot hybridization, and long-distance PCR.

Under these conditions three main *cag* PAI structures were observed: an uninterrupted *cag* PAI, no *cag* PAI, and a *cag* PAI split in two (Table 2). Nine strains did not fall into any of these three major groups: one strain had the middle region and cagII, two strains had the middle region and cagI, one strain had cagII only, one strain had cagI only, one strain had the middle region only, and three strains had the *cagA* gene only.

Of the 153 strains, 33 (21.6%) possessed an IS*605*. The distribution of IS*605*-positive strains over the different *cag* PAI structure groups is shown in Table 2. The presence of IS*605* was statistically associated with the presence of an integral *cag* PAI, either uninterrupted or split in two ($P = 0.0065$).

Correlation between the *cag* PAI and IL-8 induction. The ability of *H. pylori* to induce IL-8 secretion by eucaryotic cells was investigated by using 138 of the 153 isolates. A cutoff value of 71 pg/ml was established using the Tx30a reference strain, which is known as a non-IL-8 inducer (4). Among the 138 strains tested, 81 (58.7%) induced IL-8 secretion by the HEp-2 cells (from 71 to 4,071 pg/ml) while 57 (41.3%) induced no or low IL-8 secretion (from 0 to 71 pg/ml). The ability of a given strain to induce IL-8 secretion correlated with the structure of the *cag* PAI (Table 3). Strains possessing the entire *cag* PAI (either uninterrupted or split in two) induced IL-8 secretion significantly more often than strains with no *cag* PAI. However, no statistical difference with regard to IL-8 induction ability was observed between strains possessing an uninterupted *cag* PAI and strains with a *cag* PAI split in two. Neither the IL-8 induction ability nor the *cag* PAI structure was correlated with the clinical manifestations of the infection (Table 2).

DISCUSSION

The outcome of *H. pylori* infection is likely to be determined by a combination of factors including the virulence of the infecting strain, the host response to the infection, and several environmental cofactors. Of these factors, the virulence markers of the infecting strains have been the most studied, al-

TABLE 3. Correlations between the structure of the *cag* PAI and IL-8 induction ability

Structure of *cag* PAI	No. of IL-8 inducers/ total no. of strains (%)	Avg (SD) IL-8 production (pg/ml)
Uninterrupted	60/78 (76.9)	454.1 (751.7)
No *cag* PAI	8/40 (20)	102 (280.5)
cag PAI split in two	8/12 (66.6)	632.9 (864.5)
Other combinations	4/8 (55.5)	—[a]

[a] One strain had the middle region and cagII (IL-8 production, 10.8 pg/ml), two strains had the middle region and cagI, (137.8 pg/ml), one strain had cagII only (113.3 pg/ml), one strain had cagI only (25.2 pg/ml), one strain had the middle region only (250 pg/ml), and three strains had *cagA* only (70.45 pg/ml).

though the roles of most of them are still under discussion and many of them remain unknown. Like others, we support the hypothesis that the inflammatory response of the gastric mucosa to *H. pylori* infection is key to understanding the mechanisms leading to the severe diseases associated with the infection (11, 14, 20). Any factor resulting in an increase in gastric inflammation may also increase the risk of severe outcomes, especially gastroduodenal ulcer and gastric adenocarcinoma. In this work, the proinflammatory power of a strain was evaluated by its ability to induce IL-8 secretion from HEp-2 cells. This method has been validated previously (4, 10) and assesses for the intrinsic virulence of the strain independently of the patient response.

Previous studies showed that the *cag* PAI was rather conserved and that a few markers could be used to described it (1, 15, 20). Therefore we used a simple and global technique to investigate the structure of the *cag* PAI: the whole *cag* PAI was covered by long-distance PCR using three sets of primers. We completed this study by amplifying genes belonging to the various parts of the *cag* PAI (*virD4* for cagII, *cag13-cag14* for the middle part, and *picB* and *cagA* for cagI). The structure of the *cag* PAI appeared to be conserved, as three major groups representing 94.1% of the strains studied were observed: an uninterrupted *cag* PAI, no *cag* PAI, and a *cag* PAI split in two. This is consistent with the findings of other studies which showed that the structure of the *cag* PAI was rather conserved; Censini et al. (7), Jenks et al. (15), and Maeda et al. (20) reported that only 5 to 10% of strains had partial deletions in the *cag* PAI. In our study, 21 strains (13.7%) presented partial deletions of the *cag* PAI. Therefore, we explored the mechanisms underlying *cag* PAI partial deletion by testing for the presence of the insertion sequence IS*605*. IS*605* may be present anywhere in the *H. pylori* chromosome and is thought to be involved in rearranging the order of *H. pylori* genes (2, 26). It has been hypothesized that IS*605* was acquired by *H. pylori* later in evolutionary time than the *cag* PAI and that it is a prerequisite for *cag* deletions (7). In our study 50% of the strains that had the *cag* PAI split in two possessed an IS*605*. Although the location of the IS*605* was not determined, we can hypothesize that the separation of the two halves of the *cag* PAI could have been mediated by that transposase. On the other hand, the strains without an IS*605* underwent other mechanisms to split their *cag* PAI. This is confirmed by the fact that none of the nine strains with partial deletions of *cag* PAI possessed an IS*605*. Maeda et al. (20) and Jenks et al. (15) also found that some strains with partial *cag* PAI deletions lacked IS*605* and observed that some strains lacking the *cag* PAI possessed an IS*605* (10% in our study). Although these findings do not disprove the original explanation of *cag* deletion, they suggest that deletion without IS*605* may be possible. Further research is needed to clarify this.

From the different genes and parts of the *cag* PAI studied, the elements which appeared to be the best markers for the presence of the *cag* PAI were the *picB* and the *virD4* genes. Those two genes were better predictors than the *cagA* gene (15, 20), although none of the markers studied could predict at 100% the presence of the *cag* PAI. Because the *cag* PAI may be partially deleted or diversely organized, it is likely that the presence of one or even several genes is not sufficient to assess the presence of this region.

As was previously found by different authors, the structure of the *cag* PAI was not correlated with the clinical manifestations of the disease (14, 15, 20, 24). Our results suggest that the ability to induce IL-8 secretion by HEp-2 cells depends on the presence of an entire *cag* PAI, either uninterrupted or split in two. Although the presence of a functional *cag* PAI increases the proinflammatory power of a strain, it may have no predictive value for the presence or the future development of a clinically significant outcome, as other factors influence the evolution of the disease (14). Nevertheless, it is likely that strains with functional *cag* PAIs are more often involved in severe outcomes.

We report here the induction of IL-8 secretion by strains negative for *cag* PAI and the existence of *cag* PAI-positive strains unable to induce IL-8 secretion. This indicates that the *cag* PAI, or at least part of it, is not the only element required for IL-8 induction. Yamaoka et al. (30) found that *cag* PAI-negative strains containing a functional HP0638 gene, encoding one of the 32 outer membrane proteins detected in the genome sequence (2, 26), produced more than threefold more IL-8 than *cag*-negative strains containing a nonfunctional HP0638 gene. They therefore hypothesized that HP0638 may be an important marker of the inflammatory power of a strain and designated the HP0638 gene as encoding an outer membrane inflammatory protein (*oipA*).

The precise functions of the *cag* PAI, involved in the inflammatory power of the strain, are still not identified, although several pathways are suspected. Odenbreit et al. (22) and Stein et al. (25) showed that *cagA*-positive strains translocated the bacterial protein CagA into gastric epithelial cells using a type IV secretion system encoded by the *cag* PAI. CagA was then phosphorylated on tyrosine residues by an as yet unidentified host cell kinase and induced changes in the tyrosine phosphorylation of distinct cellular proteins. Such modifications of host cell components by bacterial protein translocation add a new dimension to the understanding of chronic *H. pylori* infection with several yet unknown consequences. Keates et al. (17) showed that *cag* PAI-positive isolates were more potent than *cag* PAI-negative strains in inducing mitogen-activated protein (MAP) kinase phosphorylation and that some gene products of the *cag* PAI were required for maximal MAP kinase activation. They also demonstrated that the p38 and MEK-1 MAP kinase activities were required for IL-8 induction by *H. pylori* but did not appear to be essential for *H. pylori*-induced NF-κB activation. Since MAP kinases regulate cell proliferation, cell differentiation, programmed cell death, reactions to stress, and inflammatory responses, activation of gastric epithelial cell MAP kinases by *H. pylori cag* PAI-positive strains may be crucial in inducing gastroduodenal inflammation, ulceration, and neoplasia. Despite these interesting results, much remains to be learned about the role of the *cag* PAI in colonization by *H. pylori*, persistence of infection, and mechanisms of associated disease.

Using an original approach, we have described the global structure of the *cag* PAI for a substantial number of clinical *H. pylori* strains. As previously shown, we observed that *cag* PAIs belong to three major structural groups and that IS*605* may play a role in determining the structural type. A few strains have *cag* PAIs with atypical structures. Such evaluation of the structure of the *cag* PAI may help to define the virulence

of a given *H. pylori* strain but is not sufficient to predict the clinical outcome of the disease.

ACKNOWLEDGMENTS

We thank the University and the CHU of Poitiers, as well as the Fondation pour la Recherche Medical, for their financial support.

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Editor: J. D. Clements