# CagA and VacA Polymorphisms Do Not Correlate with Severity of Histopathological Lesions in *Helicobacter pylori*-Infected Greek Children[∇][†]

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The presence of various numbers of EPIYA tyrosine phosphorylation motifs in the CagA protein of *Helicobacter pylori* has been suggested to contribute to pathogenesis in adults. In this prospective study, we characterized *H. pylori* isolates from symptomatic children, with reference to the diversity of functional EPIYA motifs in the CagA protein and *vacA* isotypes, and assessed the potential correlation with the histopathological manifestations of the infection. We analyzed 105 *H. pylori* isolates from 98 children and determined the diversity of EPIYA motifs in CagA by amplification and sequencing of the 3′ variable region of the *cagA* gene as well as *vacA* isotypes for the signal, middle, and intermediate regions. CagA phosphorylation and levels of secreted IL-8 were determined following in vitro infection of AGS gastric epithelial cells. Histopathological evaluation of *H. pylori* colonization, activity, and severity of the associated gastritis was performed according to the updated Sydney criteria. EPIYA A (GLKN[ST]EPIYAKVNKKK), EPIYA B (Q[V/A]ASPEPIY[A/T]QV AKKVNAKI), and EPIYA C (RS[V/A]SPEPIYATIDDLG) motifs were detected in the ABC (46.6%) and ABCC (17.1%) combinations. No isolates harboring more than two EPIYA C motifs in CagA were found. The presence of isogenic strains with variable numbers of CagA EPIYA C motifs within the same patient was detected in seven cases. Occurrence of increasing numbers of EPIYA C motifs correlated strongly with presence of a high-vacuolation (s1 or s2/i1/m1) phenotype and age. A weak positive correlation was observed between vacuolating *vacA* genotypes and presence of nodular gastritis. However, CagA- and VacA-dependent pathogenicities were not found to contribute to severity of histopathology manifestations in *H. pylori*-infected children.

*Helicobacter pylori* infects 50% of the world's population, and wide differences in prevalence of infection appear to exist between countries with different levels of socioeconomic development. Infection usually occurs in childhood and in the majority of cases remains asymptomatic, although major reasons for endoscopy referral can include recurrent epigastric or abdominal pain, with or without vomiting, neither of which correlates with *H. pylori* infection (17). Antral nodularity is a well-described endoscopic feature of *H. pylori*-infected children, and histological observations usually include superficial chronic active gastritis with occasional infiltration of eosinophils; in far fewer cases, they include peptic ulcers; and very rarely, they include gastric atrophy and intestinal metaplasia (13, 27). If the infection is left untreated, it persists through adulthood, and although it can still remain asymptomatic in the vast majority of infected hosts, *H. pylori* infection is now regarded as the most important etiological risk factor for development of gastric cancer in developed countries (28). *H.*

*pylori* pathogenesis is manifested through a combined effect of bacterial virulence factors, host genetics, and environmental factors, which orchestrate toward the development of distinct phenotypes in adults, namely, superficial asymptomatic gastritis, duodenal ulcer, and gastric cancer (3). The expression and translocation of cytotoxin-associated gene antigen (CagA), a putative *H. pylori* virulence factor, inside gastric epithelial cells by *cagA*-positive *H. pylori* strains harboring a functional type IV secretion system has been suggested to play an important role in *H. pylori* pathogenesis (22). Early epidemiological studies of adults associated the presence of the *cagA* gene with development of peptic ulcer disease (31); gastric cancer (14); and increased inflammation (35), cellular proliferation (36), and intestinal metaplasia (20) of the gastric mucosa. However, in infected children, neither *cagA* status nor any other putative *H. pylori* virulence factor has been found to correlate with clinical outcome or severity of histological manifestations. However, recent advances into the fascinating cellular biology of CagA inside the gastric epithelial cell have enhanced its reputation as a potential bacterial oncoprotein (22). Following its translocation inside the gastric epithelial cell via the type IV secretion system (32), CagA has been shown to become at least partly tyrosine phosphorylated (5, 11, 41) by Src family kinases (42, 44) on repeating 5-amino-acid glutamic-proline-isoleucine-tyrosine-alanine (EPIYA) motifs present at the C terminus of the protein. Analysis of EPIYA motifs in CagA has

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revealed considerable type variation, depending on the peptide sequence surrounding it, namely, EPIYA A (EPIYAKVNK KK), EPIYA B (EPIYAQVAKKV), or EPIYA C (EPIYATI DDLG) in isolates from Western populations or EPIYA D (EPIYATIDFD) in isolates of Asian origin. In addition, considerable variation in number of repeating EPIYA C or D motifs at the carboxyl terminus of the protein (10, 44) has been observed, and biological activity of CagA was suggested to be determined by variation in these motifs (25) in phosphorylation-dependent as well as -independent ways (23). Hence, the number and type of EPIYA phosphorylation motifs may be viewed as putative virulence determinants of CagA activity and therefore become useful clinical markers that may predict the degree of individual *H. pylori* strain virulence potential. In this context, we proposed a PCR amplification and sequencing-based strategy for accurate characterization of the number and type of EPIYA motifs of CagA in *H. pylori* clinical isolates (34).

A multifactorial role has also been attributed to the secreted VacA virulence factor (16), a protein with multiple cellular activities, as it can disrupt endocytic trafficking of host cells, promote cell death through apoptosis, suppress the local immune system, and possibly potentiate the development of ulcers (6). Although the *vacA* gene is present in all *H. pylori* strains, it contains at least three variable parts, the s region, the i region, and the m region, which encode the signal, intermediate, and middle peptides, respectively, which have all been classified as allelic types 1 and 2. The s1-or-s2/i1/m1-or-m2 and s1-or-s2/i1-i2/m1-or-m2 VacA isotypes induce, in general, high and moderate levels of vacuolation, respectively, whereas the s1-or-s2/i2/m1-or-m2 strains induce very little or no vacuolation (39). Consequently, the *vacA* s/m genotype can also be regarded as a marker of pathogenicity of individual strains (8). Moreover, phylogenetic linkage analysis studies have indicated that there may be a functional basis for the selection of *vacA* and *cagA* isotypes (50), although there is substantial distance between *vacA* loci and *cag* genes on the bacterial genome. Furthermore, the intermediate region has been associated with development of gastric cancer (39).

In the present study, we investigated the potential association of the CagA and VacA virulence factor polymorphisms with clinicopathological manifestations of the disease in symptomatic Greek children. More specifically, *H. pylori* clinical strains isolated from symptomatic children were characterized with regard to the number and type of repeating EPIYA phosphorylation motifs in CagA protein and the *vacA* signal, intermediate, and middle region genotypes. Furthermore, these clinical isolates were carefully assessed for their ability to express phosphorylated CagA as well as induce interleukin-8 (IL-8) secretion following infection of gastric epithelial cells. Finally, the potential association of such functional bacterial determinants with *H. pylori*-associated histopathology in these patients was assessed.

## MATERIALS AND METHODS

**Patients.** The study included 98 symptomatic *H. pylori*-infected children, 2 to 16 years old, who underwent upper endoscopy at the Gastroenterology Clinic of the First Department of Pediatrics of Athens University, Aghia Sophia Children's Hospital. All children were Greek in origin, and their parents had given their consent for participation in the study. The study (protocol number 19321/13.09.2007) was approved by the Hospital Scientific Committee and the Ethical Committee (transcript 23/14.11.2007). None of the patients had received non-steroidal anti-inflammatory drugs or had recently been prescribed antibiotics for the last 3 months. Three biopsy specimens were collected from the antrum for the histology, culture, and rapid urease test (CLO test), as well as one biopsy specimen from the corpus and two biopsy specimens from the duodenum for histology.

**Isolation and culture of *H. pylori*.** Antral mucosa biopsy specimens collected from the greater curvature were aseptically placed in thioglycolate medium (Oxoid, Basingstoke, United Kingdom) and were processed for *H. pylori* isolation within 2 to 4 h after endoscopy. Specimens were vigorously vortexed with addition of sterile glass beads and cultured for up to 7 days on Chalgren-Wilkins agar plates containing antibiotics (vancomycin, 10 μg/ml; trimethoprim, 10 μg/ml; polymyxin B, $10^4$ IU/liter; amphotericin B, 2 μg/ml; nalidixic acid, 10 μg/ml; bacitracin, 30 μg/ml; and fluorocytosine, 5 μg/ml) supplemented with 7% (vol/vol) horse blood and 1% (vol/vol) Vitox (Oxoid, Basingstoke, United Kingdom) under microaerophilic conditions (CampyPak Plus; Becton Dickinson, Cockeysville, MD) at 37°C. Plates were inspected daily for the presence of suspected colonies, which were initially screened for by colony morphology analysis and Gram staining and further verified by oxidase, catalase, and urease reactions. Culture sweeps, as well as individual colonies from each patient, were collected. *H. pylori* clonal relatedness within the same patient was routinely evaluated by randomly amplified polymorphic DNA (RAPD) PCR utilizing primers 1281, D14307, and D11344 (2). Multilocus sequence tagging (MLST) analysis with primers for the *atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, *vacA*, and *yphC* housekeeping genes (19) was used to further characterize isogenic *H. pylori* strains expressing CagA with the divergent number of EPIYA motifs isolated from the same patient. In total, 105 *H. pylori* clinical isolates were collected and stored in brain heart infusion broth supplemented with 20% glycerol at −80°C until further analysis.

**Characterization of diversity of EPIYA phosphorylation motifs.** Characterization of the EPIYA phosphorylation motifs was accomplished utilizing our strategy as described before (34). Briefly, each clinical isolate was passed twice on Chalgren-Wilkins plates and total bacterial genomic DNA (optical density at 260 nm/optical density at 280 nm ≥ 1.800) was extracted using a DNeasy isolation kit provided by Qiagen AS (Oslo, Norway). The EPIYA-coding regions of the *cagA* gene were amplified (EPIYA PCR) utilizing primers cagA2530S (5′-GTTAAR AATRGTGTRAAYGG-3′, where R represents A or G and Y represents T or C) and cagA3000AS (5′-TTTAGCTTCTGATACCGC-3′), which recognize positions 582453 to 582977 with reference to the *H. pylori* 26695 genome. Amplicons ranging from 370 to 670 bp (±25 bp) were visualized by agarose gel electrophoresis and sequenced using a GenomeLab DTCS Quick Start sequencing kit (Beckman Coulter, Fullerton, CA) with a CEQ 8000 Beckman Coulter genetic analyzer. The number and type of EPIYA motifs were determined from the deduced peptide sequences following alignment by CLUSTAL W (European Bioinformatics Institute [http://www.ebi.ac.uk/Tools/clustalw2/index.html]). EPIYA PCR-negative cases were confirmed as true *cagA*-negative isolates by an empty-site-positive PCR assay as described before (1).

**Detection of *vacA* gene diversity.** VacA signal region isotyping was performed by PCR using the VA1-F and VA1-R primers, and middle region isotyping was carried out by multiplex PCR utilizing primers VA4-F, VA4-R, VA7-F, and VA7-R (7). Intermediate region genotypes were determined by PCR according to the instructions of Rhead et al. (39).

**In vitro infection of gastric epithelial cells (AGS) with clinical isolates.** Human gastric adenocarcinoma epithelial AGS cells ($2 \times 10^6$ cells in 25-mm² flasks or $1 \times 10^6$ cells in six-well plates) cultured in F-12 Kaighn's medium (Gibco, Invitrogen, Ltd., Paisley, United Kingdom) containing 10% fetal bovine serum (Gibco) were infected with *H. pylori* clinical strains at a multiplicity of infection of 100 and incubated in a 5% $CO_2$ atmosphere. Total protein lysates and culture supernatants were collected at selected time points ranging from 1 to 48 h postinfection.

**Expression and functional analysis of CagA phosphorylation.** Total protein lysates from *H. pylori*-infected AGS epithelial cells were obtained in ice-cold lysis radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholate, 5 mM EDTA, 2 mM ʟ-dithiothreitol) containing protease and phosphatase inhibitor cocktails. Lysates with equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (7.5% polyacrylamide) and transferred onto polyvinylidene difluoride (Immobilon P; Millipore Corp., Bedford, MA) membranes. CagA expression was detected by Western blot analysis using an anti-CagA primary polyclonal antibody (Austral Biologicals, San Ramon, CA) followed by a secondary horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G polyclonal antibody (Jackson ImmunoResearch Europe, Ltd., Soham, Cambridgeshire, United Kingdom), detected by the ECL Plus

TABLE 1. CagA protein diversity with regard to EPIYA
phosphorylation motifs in *H. pylori* isolates from
symptomatic children

| Strain group and EPIYA status[a] | No. (%) of strains |
| --- | --- |
| *cagPAI* negative | 35 (33.3) |
| *cagPAI* defective | |
| ABC | 9 (8.5) |
| ABCC | 3 (2.8) |
| *cagPAI* functional | |
| AB | 1 (1.0) |
| ABC | 40 (38.1) |
| ABBC | 1 (1.0) |
| ABCC | 15 (14.3) |
| ACC | 1 (1.0) |
| Total | 105 (100.0) |

[a] A, B, and C refer to the EPIYA A (EPIYAKVNKKK[A/T/V/S]GQ), EPIYA
B (EPIY[A/T][Q/K]VAKKVNAKI), and EPIYA C (EPIYATIDDLG) motifs,
respectively.

chemiluminescence detection system (Amersham, GE Healthcare UK, Ltd.,
Buckinghamshire, United Kingdom). CagA tyrosine phosphorylation was eval-
uated by detection of CagA expression following immunoprecipitation of total
cell lysates with an anti-mouse monoclonal pY20 phospho-tyrosine antibody (BD
Transduction Laboratories, Franklin Lakes, NJ).

**Determination of IL-8 levels.** Culture supernatants collected at selected time
points over 48 h from in vitro *H. pylori*-infected AGS gastric epithelial cells
(multiplicity of infection, 100) were centrifuged at 13,000 rpm, and the levels of
IL-8 were determined by using a commercial enzyme-linked immunosorbent
assay kit (Bender MedSystems GmbH, Vienna, Austria) according to the man-
ufacturer's protocol.

**Histological analysis.** Biopsy specimens destined for histopathology evaluation
were fixed in 10% neutral buffered formalin solution, processed for histol-
ogy, and embedded in paraffin.

Several serial longitudinal 4-μm sections from each specimen were cut, two of
them were stained with hematoxylin-eosin for evaluation of gastric inflammation,
and one was analyzed with the May-Grünwald Giemsa method for assessment of
*H. pylori* colonization. Bacterial density and pathology of gastric mucosa were
assessed according to the updated Sydney System (18). Histopathological eval-
uation was performed by a histopathologist with no prior knowledge of the
identity of the samples.

**Statistical analysis.** Statistical analysis with reference to potential associations
between virulence factor variability and histopathological manifestations was
conducted by using the SPSS package.

**Nucleotide sequence accession numbers.** The partial *cagA* nucleotide se-
quences generated in the present study were submitted to the GenBank/EMBL/
DDBJ databases (accession numbers AM292556 and -7, AM292559 to -76,
AM292579 to -95, AM295786, AM295789, and FM957544 to -62).

## RESULTS

**Patient demographics, clinical outcomes, and histopatholo-
gies.** In total, 98 symptomatic children (49 male), aged 2 to 16
years (mean age, $10.7 \pm 0.3$ years), were included in the study.
Symptoms included epigastric or abdominal pain ($n = 73$),
pain coupled with vomiting ($n = 10$), or just vomiting ($n = 15$).
Upon endoscopic evaluation, antral nodular gastritis was evi-
dent in 72 out of 98 patients (73.5%) and edema and erythema
were evident in the corpus in 42 cases (42.7%), while in 6
children (5 male), duodenal ulcer was observed. Upon histo-
logical examination, all patients developed chronic active gas-
tritis in the antrum, and 41 patients also developed it in the
corpus. Neutrophil infiltration was assessed as mild ($n = 9$

patients), moderate ($n = 80$), or marked ($n = 7$). Mild ($n = 5$),
moderate ($n = 51$), and marked ($n = 40$) lymphocytic infiltra-
tion was also observed. The presence of eosinophils was ap-
parent in 43 patients (mild, $n = 26$; moderate, $n = 17$). *H.
pylori* colonization was classified as absent ($n = 9$), mild ($n =
10$), moderate ($n = 67$), and marked ($n = 10$). Only one patient
presented with atrophy and none with intestinal metaplasia.
Lymphoid follicle formation was observed in 27 patients (18
male). Finally, no formation of ectopic gastric mucosa in the
duodenum was observed in our sample population.

**Determination of EPIYA diversity in CagA protein.** Ninety-
eight *H. pylori* clinical isolates were collected from our patients,
and the bacterial DNA was subjected to EPIYA PCR for
determination of EPIYA diversity. Thirty-five isolates (33.3%)
were found to be negative in EPIYA PCR amplification (Table
1). These were all confirmed as true *cagPAI*-negative strains by
empty-site positive PCR (1), which generates an amplicon in
the absence of the whole pathogenicity island (data not
shown). They were further verified by the absence of an im-
munospecific band in Western blot determination of CagA
expression in individual protein lysates. In 56 isolates, single-
band amplification by EPIYA PCR (Fig. 1A) and CagA pro-
tein expression (Fig. 1B) was observed. However, in the iso-
lates from seven patients, we observed two amplified bands in
the EPIYA-PCR (Fig. 1A). We verified by isolation and se-
quencing that these were *cagA*-specific gene sequences, indi-
cating the presence of at least two infecting strains within the
same patient. The expression of such dual CagA protein spe-
cies was further verified by Western blot analysis of total pro-
tein lysates of AGS cells infected with the corresponding *H.
pylori* strains (Fig. 1B), indicative of the simultaneous presence
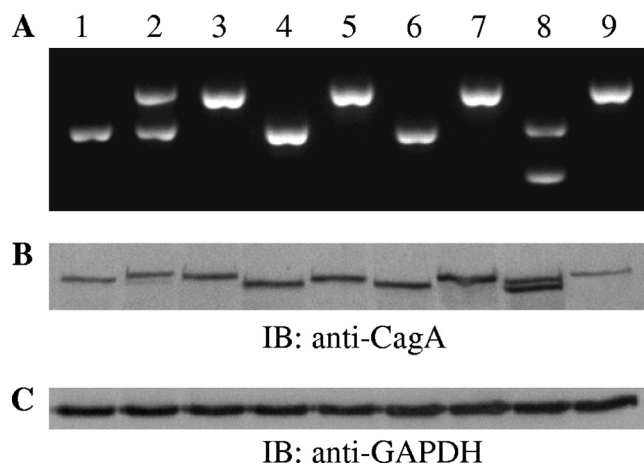of two infecting strains expressing CagA protein with divergent



FIG. 1. CagA protein polymorphism with reference to EPIYA mo-
tifs. DNA from representative *H. pylori* clinical strains (lanes 1 to 9)
was amplified by EPIYA PCR, and the PCR products were analyzed
on a 1.5% agarose gel (A). Analysis of the expressed CagA proteins by
immunoblotting (IB) (B) with anti-CagA rabbit polyclonal antibody,
following in vitro infection of AGS gastric epithelial cells with the
corresponding isolates shown in panel A. Note the presence of two
amplified bands in samples 2 and 8 in panel A, denoting the presence
of two distinct amplicons and the expression of the respective two
different CagA protein species (B). The immunoblot in panel C depicts
expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase)
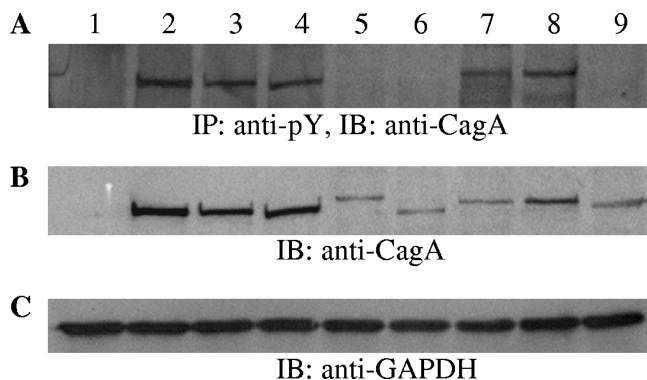protein as a total protein loading control for panel B.

FIG. 2. Phosphorylation and expression of CagA protein following infection of gastric epithelial cells with representative *H. pylori* clinical isolates. CagA tyrosine phosphorylation (A) was evaluated by immunoblotting (IB) with anti-CagA rabbit polyclonal antibody following immunoprecipitation (IP) of total cell lysates with an anti-mouse monoclonal pY20 phospho-tyrosine antibody. CagA expression (B) was determined by immunoblotting with anti-CagA rabbit polyclonal antibody on total cell lysates. Lane 1, *cagPAI*-negative isolate. Lanes 2 to 4 and 7 and 8, isolates with functional *cagPAI* and CagA protein with three (ABC) and four (ABCC) EPIYA motifs, respectively. Lanes 5, 6, and 9, *cagA*-positive isolates with defective *cagPAI* resulting in the absence of CagA protein phosphorylation. The immunoblot in panel C depicts expression of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein as a total protein loading control for panel B. Anti-CagA rabbit polyclonal antibody and anti-GAPDH mouse monoclonal antibody were utilized at 1:4,000 and 1:10,000 dilutions, respectively, in the presence of 5% nonfat dry milk. Protein separation by SDS-polyacrylamide gel electrophoresis was realized using 6% polyacrylamide gels.
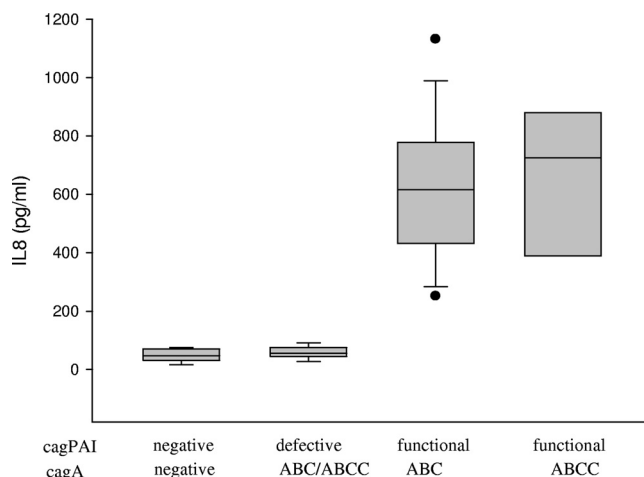


FIG. 3. Levels of IL-8 secreted by gastric epithelial cells following 48-hour in vitro infection with *H. pylori* clinical isolates. *H. pylori* strains are classified as *cagPAI* negative, *cagPAI* defective, and *cagPAI* functional, with the CagA protein harboring EPIYA motifs in the ABC and ABCC combinations.

numbers of EPIYA motifs. We proceeded to separate these pairs of isolates by following the methodology described in our earlier publication (34), bringing the total number of strains to 105. More specifically, we successfully separated those subclones by limiting dilution, *H. pylori* colony selection, and screening of individual colonies for a single PCR amplicon by our EPIYA PCR. RAPD PCR analysis on genomic DNA derived from the isolated pairs of strains revealed identical profiles (see Fig. S1 in the supplemental material), an indication that these strains were clonally closely related. The subclones were further subjected to MLST analysis, which afforded identical sequences for all seven genes (data not shown), suggesting that the isolated clones were isogenic. For six out of seven patients, sequence analysis of the EPIYA PCR amplicons revealed the simultaneous presence of CagA protein species with either three or four EPIYA domains (ABC/ABCC combination). Upon comparison, sequences were found to be identical on a nucleotide basis outside the 102-bp sequence repeat coding for the 34-amino-acid peptide segment containing the additional EPIYA C motif. The remaining isolate contained two subclones expressing CagA with either an AB or an ABC combination of EPIYA motifs.

The nucleotide sequences of the EPIYA-PCR amplicons from all 105 *H. pylori* isolates were aligned, and the deduced peptide sequences revealed the presence of EPIYA motifs, namely, EPIYA A (GLKN[ST]EPIYAKVNKKK), EPIYA B (Q[V/A]ASPEPIY[A/T]QVAKKV), and EPIYA C (RS[V/A]SPEPIYATIDDLG). The ST dipeptide preceding the EPIYA A motif was observed in only 15 patients. The majority of

isolates contained alanine (A) ($n = 46$) within the EPIYA B motif; however, equal distributions of valine and alanine were observed for EPIYA C. No significant variations in the individual peptide sequences surrounding the EPIYA motifs were observed in our sample from children and those already published for adult populations in Greece (34). In all, 49 out of 105 (46.7%) isolates were found to possess three EPIYA domains in the ABC conformation and 18 (17.1%) isolates four domains in the ABCC conformation (Table 1). Rare cases of CagA proteins with EPIYA domains in combinations such as ABBC ($n = 1$) and ACC ($n = 1$) were also observed, whereas no cases with more than two EPIYA C repeats were detected. For the *cagA*-positive isolates, *cagPAI* functionality was assessed by determination of CagA protein phosphorylation (Fig. 2) and levels of secreted IL-8 in the supernatant (Fig. 3), following incubation of *H. pylori* strains with AGS gastric epithelial cells. In this way, we identified 12 isolates with defective *cagPAI* within our *cagA*-positive population, 9 with the ABC and 3 with the ABCC combination of EPIYA (Table 1).

**Classification of strains according to *cagA* EPIYA diversity and *cagPAI* status.** In view of the critical role of *cagPAI*-encoded proteins in successful translocation of CagA protein inside the gastric epithelial cell and the putative role of phosphorylated EPIYA C motifs in increased deregulation of SH2 domain-containing protein-tyrosine phosphatase-2 (SHP2 phosphatase) (24), we grouped our isolates according to the presence of phosphorylated CagA protein as well as the number of EPIYA C motifs. Consequently, *cagA*-negative strains, as well isolates for which we could not detect phosphorylated CagA, were grouped together ($n = 48$ isolates) (Tables 2, 3, and 4). In this group, a *cagA*-positive strain without EPIYA C motifs (AB) was also included. Forty-one isolates bearing three EPIYA motifs in an ABC combination and one isolate with the ABBC combination of EPIYA motifs, all with functional *cagPAI*, were classified in the group with one EPIYA C motif. Accordingly, 16 isolates with CagA bearing four EPIYA motifs (ABCC) as well as 1 isolate with the ACC combination and

TABLE 2. CagA protein diversity with regard to EPIYA-C motifs and *vacA* signal, intermediate, and middle region isotypes

| VacA isotype | No. of isolates with indicated no. of EPIYA C repeats[a] in CagA protein (%) | | |
|---|---|---|---|
| | Zero | One | Two |
| s1 | | | |
| i1/m1 | 3 (2.9) | 11 (10.5) | 6 (5.7) |
| i1/m2 | 4 (3.8) | 3 (2.9) | 2 (1.9) |
| i1-i2/m1 | 2 (1.9) | 7 (6.7) | 0 |
| i1-i2/m2 | 6 (5.7) | 7 (6.7) | 2 (1.9) |
| i2/m1 | 0 | 1 (1.0) | 0 |
| i2/m2 | 4 (3.8) | 9 (8.6) | 4 (3.8) |
| s2 | | | |
| i1/m2 | 0 | 1 (1.0) | 1 (1.0) |
| i1-i2/m2 | 10 (9.5) | 2 (1.9) | 1 (1.0) |
| i2/m2 | 19 (18.1) | 0 | 0 |

[a] The presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "Zero" refers to the absence of tyrosine-phosphorylated CagA.

functional *cagPAI* were classified in the group with two EPIYA C motifs. No correlation was observed between EPIYA C diversity and gender of host (Pearson's $\chi^2 = 0.787$; $P = 0.675$). Finally, no correlation was observed between EPIYA C status and reason for referral (Pearson's $\chi^2 = 2.773$; $P = 0.597$), as the overwhelming majority of our patients were referred for recurrent abdominal pain.

**Determination of *vacA* diversity and correlation with *cagA* genotype.** We characterized all isolates according to the *vacA* signal, intermediate, and middle region isotypes (Table 2). In the absence of functional EPIYA C motifs (Table 2), the majority of isolates (41 out of 48) were found to possess presumed nonvacuolation (s2/i2/m2 [$n = 19$; 18.1%] and s1/i2/m2 [$n = 4$; 3.8%]) or low-vacuolation (s1/i1-i2/m1 or m2 [$n = 8$; 7.6%] and s2/i1-i2/m2 [$n = 10$; 9.5%]) isotypes, whereas only 7 (6.7%) isolates were of high-vacuolation (s1/i1/m1 or m2) isotypes. No isolates bearing the s2/m1 isotype, irrespective of intermediate region type, were observed. In the overwhelming majority of CagA-positive strains with functional *cagPAI*, the presence of EPIYA C motifs was positively correlated (Pearson chi-square test; $P < 0.001$) with high-vacuolation (s1/i1/m1 or m2 [$n = 22$; 21.0%]) or lower-vacuolation (s1/i2 or i1-i2/m1 or m2 [$n = 30$; 28.8%]) isotypes. Only five (4.9%) among the *cagA*-positive isolates were found to be of nonvacuolation (s2/i1 or i2 or i1-i2/m2) isotypes. A positive correlation between presence of high-vacuolation isotypes and increasing age was observed (Pearson chi-square test; $P = 0.026$) (Table 3). Interestingly, in 12 out of 17 cases where two EPIYA C motifs were present (including cases of mixed ABC/ABCC infection), the patients

were over 10 years old (Table 3). Therefore, a positive correlation between higher number of EPIYA C motifs in the CagA protein and increasing age may also exist, but analyses of more cases are required for determination of this.

**Association of EPIYA diversity in CagA protein and *vacA* isotypes with histopathology in the antrum.** To facilitate statistical analysis, isolates were classified according to (i) the numbers of EPIYA C motifs in the groups, namely none, one, or two, and (ii) the levels of vacuolating potential, namely, high for s1/i1/m1-or-m2 isotypes, low for s1-or-s2/i1-i2 isotypes, and none for s1-or-s2/i2 isotypes, irrespective of the middle region isotype. This classification was utilized for the assessment of possible associations with the corresponding histological manifestations in the 91 patients where infection by a single *H. pylori* strain was detected (Table 4). No correlation was observed between number of repeating EPIYA motifs and either chronic gastritis activity, chronic inflammatory infiltration, or levels of *H. pylori* colonization in the antrum in the children in our study ($P = 0.571$, $P = 0.193$, and $P = 0.074$, respectively) (Table 4). Also, no correlation with grade of eosinophil infiltration ($P = 0.957$) or presence of lymphoid follicle formation ($P = 0.912$) was observed (Table 5). Furthermore, with reference to the *vacA* genotype, we did not observe any correlation with chronic gastritis activity, chronic inflammatory infiltration, or levels of *H. pylori* colonization in the antrum ($P = 0.054$, $P = 0.499$, and $P = 0.230$, respectively) (Table 4) or with eosinophil infiltration or formation of lymphoid follicles ($P = 0.341$ and $P = 0.283$, respectively) (Table 5). Interestingly, the only significant correlation was the one between *vacA* genotype and presence of nodular gastritis (Pearson chi-square test; $P = 0.034$) (Table 6), an endoscopic observation quite common in cases of *H. pylori*-associated gastritis in children. All correlations between EPIYA C status and different *vacA* isotypes and clinical and histopathological manifestations in the antrum were found to be nonsignificant. Collectively, these results suggest that the number of repeating EPIYA C motifs in CagA protein, coupled with the distinct *vacA* isotypes, does not seem to correlate with severity of gastric inflammation in *H. pylori*-infected children.

**Induction of IL-8 levels and relation to virulence factor variability.** In order to evaluate whether potential differences in IL-8 level depended upon EPIYA diversity, we infected AGS cells in vitro over a 48-h period with *H. pylori* isolates (i) without *cagPAI* ($n = 18$), (ii) with *cagPAI* present but defective ($n = 10$), and (iii) with CagA positivity and functional *cagPAI* and EPIYA motifs in the ABC ($n = 19$) or ABCC ($n = 7$) combination and determined the levels of IL-8 protein secreted in the supernatant. No significant difference in IL-8

TABLE 3. VacA and CagA diversity in relation to patient age

| Patient age | No. of isolates with indicated vacuolation level[a] (%) | | | No. of isolates with indicated no. of EPIYA C repeats[b] in CagA protein (%) | | |
|---|---|---|---|---|---|---|
| | None | Low | High | Zero | One | Two |
| ≤10 yr | 19 (19.4) | 16 (16.3) | 5 (5.1) | 20 (20.4) | 15 (15.3) | 5 (5.1) |
| >10 yr | 17 (17.3) | 18 (18.4) | 23 (23.5) | 26 (26.5) | 20 (20.4) | 12 (12.2) |

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and nonvacuolation (s1 or s2/i2) groups, irrespective of middle region status.
[b] The presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "Zero" refers to the absence of tyrosine-phosphorylated CagA.

TABLE 4. CagA and VacA diversity among *H. pylori* isolates in relation to colonization levels and associated gastritis in the antrum

| VacA isotype and no. of EPIYA C repeats[a] | No. of patients[b] with: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *H. pylori* colonization | | | | Chronic gastritis activity | | | Chronic inflammatory infiltration | | |
| | Normal | Mild | Moderate | Marked | Mild | Moderate | Marked | Mild | Moderate | Marked |
| High vacuolation | | | | | | | | | | |
| 0 | 1 | 1 | 4 | 0 | 0 | 6 | 0 | 0 | 4 | 2 |
| 1 | 1 | 1 | 7 | 3 | 0 | 10 | 2 | 0 | 5 | 7 |
| 2 | 1 | 3 | 2 | 0 | 0 | 6 | 0 | 0 | 6 | 0 |
| Low vacuolation | | | | | | | | | | |
| 0 | 0 | 3 | 13 | 1 | 2 | 15 | 0 | 0 | 10 | 7 |
| 1 | 0 | 0 | 11 | 2 | 0 | 13 | 0 | 0 | 8 | 5 |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| No vacuolation | | | | | | | | | | |
| 0 | 2 | 2 | 16 | 3 | 4 | 17 | 2 | 3 | 12 | 8 |
| 1 | 1 | 0 | 8 | 0 | 2 | 6 | 1 | 1 | 1 | 7 |
| 2 | 1 | 0 | 2 | 0 | 0 | 2 | 1 | 0 | 0 | 3 |

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and no-vacuolation (s1 or s2/i2) groups, irrespective of middle region status. For each isotype, the presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "0" refers to the absence of tyrosine-phosphorylated CagA.
[b] The severity levels (normal, mild, moderate, and marked) are defined in reference 18.

levels was observed between *cagA*-positive isolates with one and two EPIYA C motifs in the CagA protein (mean levels of 615.5 ± 51.6 pg/ml and 724.9 ± 89.7 pg/ml, respectively) (Fig. 3). As expected CagA-negative isolates lacking functional *cagPAI* induced only basal IL-8 levels ($n = 12$ isolates; 48.4 ± 7.7 pg/ml). Within the CagA-positive population tested, 62.8% ($n = 22$) of the isolates were positive for the presence of phosphorylated CagA protein and induction of IL-8 secretion. Finally, we sought to determine the potential difference in level of secreted IL-8 between the individual isogenic subclones expressing CagA protein with variable numbers of EPIYA C repeats isolated from the same patient. AGS cells were in-

fected over a 48-h period with the individual *H. pylori* isogenic subclones expressing CagA with EPIYA repeats in the AB or ABC combination (GenBank accession numbers AM292594 and AM292593) and a second pair of subclones with the ABC or ABCC combination (GenBank accession numbers AM292577 and AM292578). No differences in level of secreted IL-8 were observed between the two isogenic subclones expressing CagA with EPIYA in the AB or ABC combination (1,331.5 ± 129.0 versus 1,319.0 ± 128.2 pg/ml) or between the two subclones with the ABC or ABCC combination (1,384.3 ± 101.6 pg/ml versus 1,242.7 ± 112.2 pg/ml).

Consequently, no correlation between level of secreted IL-8 and EPIYA combination was observed. Also, no correlation was observed between IL-8 level and chronic gastritis activity, chronic inflammatory infiltration, or level of *H. pylori* colonization in the antrum (data not shown).

## DISCUSSION

In the present study, we have examined the potential correlation between *H. pylori* virulence factor diversity and endoscopic and histopathologic manifestations in symptomatic children. With relation to the CagA protein, EPIYA phosphorylation motif diversity has been suggested to be a major contributor to *H. pylori* pathogenesis in adults (12). From a mechanistic point of view, CagA has been suggested to interact in a

TABLE 5. CagA and VacA diversity among *H. pylori* isolates in relation to eosinophil infiltration and lymph node hyperplasia in the antrum

| VacA isotype and no. of EPIYA C repeats[a] | No. of patients with: | | | | |
|---|---|---|---|---|---|
| | Eosinophil infiltration[b] | | | Lymph node hyperplasia | |
| | Mild | Moderate | Marked | No | Yes |
| High vacuolation | | | | | |
| 0 | 2 | 2 | 2 | 5 | 1 |
| 1 | 6 | 5 | 1 | 8 | 4 |
| 2 | 2 | 2 | 2 | 6 | 0 |
| Low vacuolation | | | | | |
| 0 | 9 | 6 | 2 | 14 | 4 |
| 1 | 7 | 4 | 2 | 10 | 3 |
| 2 | 0 | 0 | 0 | 0 | 0 |
| No vacuolation | | | | | |
| 0 | 14 | 5 | 4 | 15 | 8 |
| 1 | 7 | 0 | 2 | 6 | 3 |
| 2 | 2 | 1 | 0 | 1 | 2 |

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and nonvacuolation (s1 or s2/i2) groups, irrespective of middle region status. For each isotype, the presence of the characteristic EPIYA C motif (EPIYATIDDLG) is indicated. "0" refers to the absence of tyrosine-phosphorylated CagA.
[b] The severity levels (mild, moderate, and marked) are defined in reference 18.

TABLE 6. VacA isotypes and endoscopic observation of nodular gastritis

| Nodular gastritis status | No. (%) of isolates with indicated vacuolation level[a] | | |
|---|---|---|---|
| | High | Low | None |
| Absent | 9 (9.9) | 3 (3.3) | 12 (13.2) |
| Present | 16 (17.6) | 28 (30.8) | 23 (25.3) |

[a] VacA isotypes were classified into high-vacuolation (s1/i1), low-vacuolation (s1 or s2/i1-i2), and nonvacuolation (s1 or s2/i2) groups, irrespective of middle region status.

phosphorylation-dependent manner with and perturb the normal activity of the tyrosine phosphatase SHP2 (26), the C-terminal Src kinase Crk (47), and the Crk adaptor proteins (45). Deregulation of SHP2 and its downstream effector focal adhesion kinase (48) was suggested to be mediated via the EPIYA C motif and, for the C-terminal Src kinase (Csk), via the EPIYA A or EPIYA B motif (29). In this respect, careful characterization of clinical isolates with reference to their ability to express functional CagA and determination of EPIYA diversity could provide useful predictive tools for *H. pylori* pathogenesis. Nearly 47% of *cagA*-positive isolates from the population of children analyzed in this study harbored three EPIYA motifs in the ABC combination, and only 17% harbored four EPIYA domains in the ABCC combination. Furthermore, among *cagA*-positive strains, about 19% (12 out of 63) were found to lack the capacity to express functional CagA protein and induce IL-8 secretion following infection of gastric epithelial cells. No significant variations in the individual peptide sequences surrounding the EPIYA motifs were observed in our sample from children and those already published for adult patients in Greece (34). However, although EPIYA phosphorylation motifs of the CagA protein in *H. pylori* strains isolated from Greek children do not present structural differences compared to those prevailing in adults, some subtle variations were noted. We observed a trend toward higher prevalence of *cagA* negativity in strains isolated from children than in those isolated from adults, an observation that has already been reported for populations with much higher prevalences of *H. pylori* infection (40). The higher prevalences of *cagA*- and *cagPAI*-negative *H. pylori* strains among children may reflect an evolutionary modification permitting successful colonization during transmission. Indeed, studies involving murine models (37) have shown that *H. pylori* strains in which *cagPAI* is absent or in which there is loss of functions required for activation of NF-κB exhibit a selective advantage for colonization of the murine host. Therefore, there may be a selective pressure for colonizing *H. pylori* strains to lose such features in vivo. Furthermore, we observed a positive correlation between number of EPIYA C motifs in CagA and age; in 12 out of 17 cases where two EPIYA C motifs were present (including mixed ABC/ABCC), the patients were over 10 years old. In contrast to what was found for the *H. pylori* strains isolated from adults (34), we did not detect any CagA-positive isolates harboring more than two EPIYA C repeats in children, and this may be related to reduced rates of survival of such strains in acidic conditions (49). As far as the contribution of CagA to *H. pylori* pathogenesis is concerned, this fact may offer a good explanation for the reduced severity levels of clinical and pathological manifestations associated with *H. pylori* infection in children compared to the levels observed in adults. Interestingly, we detected simultaneously present microevolving strains in the antrum with identical RAPD and MLST profiles, expressing CagA proteins with variable numbers of EPIYA motifs, including one pair with AB/ABC combinations and six pairs with ABC/ABCC combinations of EPIYA motifs in CagA. This may suggest that children when first acquiring the infection may be colonized by multiple *H. pylori* variants which, over time, through selection influenced by host genetics and bacterial factors, result in various *H. pylori* genotypes that predominate in adulthood. Similar results suggesting that, after infection in childhood, individual strains will undergo evolutionary changes during the course of infection have been reported before (33).

With relation to *vacA* status, we have observed an association between *cagA*-negative genotype and nonvacuolation (s2/i1 or i2/m2) *vacA* isotypes. Also, *cagA*-positive strains with one or two EPIYA C repeats were mostly associated with high-vacuolation (s1/i1/m1) or lower-vacuolation (s1 or s2/i1-i2) isotypes, suggesting that *cagA* and *vacA* genotypes may not be regarded as independent variables in *H. pylori* pathogenesis. Indeed, there is increasing evidence that a functional association between *vacA* and *cagA* virulence factors may exist (4). In line with this observation, isolates from our pediatric population with high vacuolation potential and higher numbers of EPIYA C motifs in the CagA protein were more prevalent among children over 10 years old.

Induction of IL-8 secretion following *H. pylori* infection of gastric epithelial cells is largely regarded to be a *cagPAI*-dependent phenomenon (43). However, there is mounting evidence that translocated CagA protein may also contribute to IL-8 induction through a Ras/Raf/Mek/extracellular signal-regulated kinase/NF-κB signaling pathway (15). We determined levels of secreted IL-8 in the supernatant of gastric epithelial cells, infected in vitro with a variety of strains, including *cagA*-negative as well as *cagA*-positive *H. pylori* isolates bearing one or two EPIYA C repeats in the CagA protein, with or without functional *cagPAI*. In this context, apart from IL-8 secretion, *cag* pathogenicity island functionality was further established by simultaneously screening for the presence of phosphorylated CagA protein in protein lysates from *H. pylori*-infected AGS cells. We observed no correlation between number of repeating EPIYA C motifs and level of proinflammatory IL-8 secreted by gastric epithelial cells. IL-8 levels were markedly different between patients, irrespective of the number of EPIYA motifs in CagA, an observation also reported by others (30, 38). Furthermore, to account for unknown variability between the different clinical strains, we utilized pairs of isogenic strains with variable numbers of EPIYA C, one of which totally lacked EPIYA C motifs. We observed no differences in level of secreted IL-8 between those isogenes with zero, one, or two EPIYA C repeats, suggesting that the contribution of the number of EPIYA C repeats in the CagA protein to IL-8 secretion may be marginal.

Although our analysis was considerably thorough, in order to characterize strains with reference to the functional status of CagA and VacA virulence factors and not just the genotypic status, no correlation between virulence factor diversity and clinical symptoms was observed, as the overwhelming majority of our patients were referred for endoscopy with symptoms of recurrent abdominal pain. Also, no correlation between virulence factor diversity and developing histopathology in the antrum and the fundus in children was observed. Similar conclusions with reference to the presence of CagA and the signal and middle region genotypic variation of VacA have been reported before in a number of studies involving *H. pylori*-infected children (9, 21, 33), but never associating functional characteristics which contribute to the pathogenic role of CagA, *cagPAI* functionality, or the more recently reported intermediate region VacA isotypes. Interestingly, the only positive, yet weak, correlation was the one observed between vac-

uolating *vacA* genotypes and presence of nodular gastritis, an endoscopic observation quite common in cases of *H. pylori*-associated gastritis in children. Such positive correlation was not observed in earlier studies involving *H. pylori* bacterial genotypes and endoscopic observations in children and may warrant further verification. *H. pylori* prevalence in biopsied symptomatic children in Greece is estimated around 14% on the basis of detection by culture or histology (E. Roma-Giannikou, unpublished data), which is in line with what is observed in other northern and western European countries (46). The lack of correlation between *H. pylori* virulence factor diversity and severity of histopathology in symptomatic children may correlate with differences in the course of infection, possibly with correlations becoming stronger as the duration of infection increases and potential increments in the number of repeating EPIYA motifs in CagA occur throughout adulthood. This is further supported by our observations that potentially more virulent forms of *H. pylori* were found to be present in older children and that CagA species with three or even four EPIYA C repeats are exclusively observed in strains isolated from adults and not from children.

## ACKNOWLEDGMENTS

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