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Pediatric *Helicobacter pylori* Isolates Display Distinct Gene Coding Capacities and Virulence Gene Marker Profiles[▽]†

Sarah Talarico,[1] Benjamin D. Gold,[2] Jutta Fero,[1] Dexter T. Thompson,[2] Jeannette Guarner,[3,4] Steven Czinn,[5] and Nina R. Salama[1]*

Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington[1]; Department of Pediatric Gastroenterology and Nutrition[2] and Department of Pathology and Laboratory Medicine,[3] Emory University School of Medicine, Atlanta, Georgia; Children's Healthcare of Atlanta, Atlanta, Georgia[4]; and Department of Pediatrics, University of Maryland, Baltimore, Maryland[5]

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***Helicobacter pylori* strains display remarkable genetic diversity, and the presence of strains bearing the toxigenic *vacA* s1 allele, a complete *cag* pathogenicity island (PAI), *cagA* alleles containing multiple EPIYA phosphorylation sites, and expressing the BabA adhesin correlates with development of gastroduodenal disease in adults. To better understand the genetic variability present among pediatric strains and its relationship to disease, we characterized *H. pylori* strains infecting 47 pediatric North American patients. Prevalence of mixed infection was assessed by random amplified polymorphic DNA analysis of multiple *H. pylori* clones from each patient. Microarray-based comparative genomic hybridization was used to examine the genomic content of the pediatric strains. The *cagA* and *vacA* alleles were further characterized by allele-specific PCR. A range of EPIYA motif configurations were observed for the *cagA* gene, which was present in strains from 22 patients (47%), but only 19 (41%) patients contained a complete *cag* PAI. Thirty patients (64%) were infected with a strain having the *vacA* s1 allele, and 28 patients (60%) had the *babA* gene. The presence of a functional *cag* PAI was correlated with ulcer disease ($P = 0.0095$). In spite of declining rates of *H. pylori* infection in North America, at least 11% of patients had mixed infection. Pediatric strains differ in their spectrum of strain-variable genes and percentage of absent genes in comparison to adult strains. Most children were infected with *H. pylori* strains lacking the *cag* PAI, but the presence of a complete *cag* PAI, in contrast to other virulence markers, was associated with more severe gastroduodenal disease.**

It is estimated that >50% of the world's population is colonized with *Helicobacter pylori* in the stomach, making it one of the most common bacterial pathogens of humans. *H. pylori* infection is generally acquired in childhood (24, 33) and can persist for life. Gastritis (inflammation of the gastric mucosa) results in all who are colonized with *H. pylori*, but some hosts remain asymptomatic, while others develop peptic ulcers, gastric adenocarcinomas, and mucosa-associated lymphoid tissue lymphoma. Gastric cancer is the second leading cause of cancer death worldwide, and 63% of gastric cancer cases in 2002 were attributable to *H. pylori* infection (38, 49). While severe disease most often presents in adulthood, children display *H. pylori*-associated gastritis and the incidence of ulcer disease among infected children was 6.8% in a European pediatric population (31). Many studies have examined bacterial, host, and environmental risk factors associated with development of *H. pylori*-associated diseases in adults, but similar studies in children have been limited.

Genetic differences among *H. pylori* strains contribute to differences in disease outcome among infected individuals in adult populations. The gene encoding VacA, which induces vacuolation of host cells, is present in nearly all *H. pylori* strains, but a number of allele types have been defined. Strains having the type s1 *vacA* signal sequence and the m1 *vacA* middle region allele (*vacA* s1/m1) are associated with ulcer disease (9). The *cag* pathogenicity island (PAI) encodes a type IV secretion system (T4SS) (1, 15) that translocates the CagA protein effector, also encoded in the island, into host cells. Presence of the *cag* PAI is associated with increased inflammation, promoting host cell interleukin-8 (IL-8) production, and *cagA*-positive strains are associated with peptic ulcers (50) as well as gastric cancer (13). Inside the host cell, CagA protein becomes tyrosine phosphorylated at C-terminal EPIYA (Glu-Pro-Ile-Tyr-Ala) sites by src family kinases, deregulates SHP-2, and induces the hummingbird phenotype (26, 45). Strains having more C-type EPIYA motifs, the major phosphorylation site, induce stronger effects on host cells and are associated with gastric cancer (7, 12, 35). The presence of a functional allele of *babA*, a gene encoding an adhesin that mediates binding to Lewis B antigens expressed on gastric epithelial cells, is associated with duodenal ulcer and gastric adenocarcinoma (21).

While these *H. pylori* genes and alleles have been associated with disease outcome in adults, studies in children have provided mixed results. A recent study identified two genes (jhp0562, coding for a putative glycosyltransferase, and jhp0870, coding for an outer membrane protein) associated with peptic ulcer disease in children, but not adults, suggesting a different spectrum of genetic risk factors in adults and children (37). Studies using a whole-genome microarray-based approach

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N, Mailstop C3-168, Seattle, WA 98109-1024. Phone: (206) 667-1540. Fax: (206) 667-6524. E-mail: nsalama@fhcrc.org.

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have been done to investigate the variability in genomic content of *H. pylori* strains, but these studies have included mostly strains from adult patients (25, 29, 41, 42). Studies of the genetic variability of pediatric *H. pylori* strains have largely been limited to genes previously associated with virulence in adult populations. To better understand the genetic variability present among pediatric strains, we used whole-genome microarray-based comparative genomic hybridization to examine the genomic content of *H. pylori* strains isolated from symptomatic North American children and compared the pediatric isolate genetic variability to that observed in adult strains. We then examined the frequency of known virulence genes and virulence alleles among the pediatric *H. pylori* strains and the associations of strain genotype with the clinical and histological characteristics of the patients.

MATERIALS AND METHODS

Patient population and endoscopic evaluation. Patients were selected randomly from subjects for whom a *H. pylori* culture was available for genotyping from three primary centers (Miami Children's Hospital, Miami, FL; Rainbow Babies & Children's Hospital, Cleveland, OH; and Children's Healthcare of Atlanta at Egleston, Atlanta, GA) and subjects referred to the primary centers from additional U.S. and Canadian Centers. Gastric biopsies were obtained during a diagnostic fiber-optic upper endoscopy performed at the discretion of the pediatric gastroenterologist because of the subjects' persistent gastrointestinal symptoms. The study cohort was accrued over a 3-year period, and patients were selected for analysis using a random numbering scheme. All patients were treated with eradication therapy. Disease diagnoses were defined as follows: normal gross appearance, erosions, ulcers, and nodularity. The study protocols and procedures for the protection of human subjects were approved by the Institutional Review Board of Emory University.

***H. pylori* isolation and histopathologic evaluation.** One biopsy from the antrum was collected and frozen for *H. pylori* isolation, and separate biopsies from the antrum and/or fundus were fixed in formalin and processed for histology at each clinical center. Histologic preparations and frozen biopsies were shipped to Emory University for histopathologic review and processing, respectively. For histopathologic evaluation, hematoxylin-and-eosin-stained slides were graded using the visual analog scale of the Sydney classification, which guided analysis of the density of *H. pylori*, and the amounts of neutrophils, mononuclear inflammatory cells, and intestinal metaplasia (19). Biopsy samples were cultured as described previously (22). The primary growth of each biopsy site was pooled and frozen in brain heart infusion broth containing 10% fetal bovine serum, 20% glycerol, and 0.2% β-cyclodextrin for storage at −80°C. For single-colony clone isolation, dilutions of the frozen primary growth were plated to obtain well-separated colonies. Individual colonies were then amplified by growth on horse blood medium (43) and frozen as individual clone stocks, and genomic DNA was prepared (Wizard Prep; Promega).

RAPD-PCR fingerprinting. Random amplified polymorphic DNA (RAPD) fingerprinting was performed as previously described (2) with primers 1254 and 14216. PCR products were electrophoresed in 1% agarose gels for comparison of banding patterns.

aCGH. Each strain was examined by a two-color array competitive genomic hybridization (aCGH) with a reference sample containing an equal molar mixture of the two sequenced strains 26695 and J99 used to design the probes on the microarray as described previously (41). Each isolate was analyzed on at least two microarrays. Data extraction and processing were performed as previously described (42), and data were simplified into a binary score for gene presence (score of 1) and absence (score of 0). Gene calls are given in Table S2 in the supplemental material.

PCR genotyping of *vacA* and *cagA* alleles. *vacA* signal sequence and mid-region PCR typing was performed as described by Atherton et al. (9, 10). The numbers and types of EPIYA motifs present in *cagA* were determined as described previously (8).

Coculture experiments. The human gastric adenocarcinoma cell line AGS (ATCC CRL-1739) was cocultured with *H. pylori* strains for analysis of IL-8 release and CagA translocation at 24 h as described previously (42).

Reference strains. The control strains were G27 (*cag* PAI$^+$ *vacA* s1a/m1) (18), Tx30a (ATCC 51932) (*cag* PAI$^-$ *vacA* s2/m2), 26695 (*cagA* ABC EPIYA) (48), and J99 (*cagA* BC EPIYA) (4).

Statistical analysis. Tests of association between different clinical characteristics, patient demographics, and strain genotypes were performed using either the Fisher's exact test or the Spearman rank correlation, as appropriate. The genomic content of the pediatric strains, based on the aCGH, was compared to that of 71 *H. pylori* strains isolated from adult patients for which aCGH had been previously performed and data reported (25, 41). Differences in the mean percentages of genes absent between the pediatric strains and the adult strains were assessed by *t* test. Because the arrays include all the genes present in strains 26695 and J99, data from these 2 of the 15 strains reported by Salama et al. (41) were removed from the analysis of the difference in mean percentages of genes absent. The relative proportions of strain-variable genes in each functional class that were variable in only the adult strains were analyzed by chi-square or the Fisher's exact test, as appropriate. Because the comparison of the pediatric array data and the adult array data could be potentially biased by including globally representative adult strains and pediatric strains from only North American patients, the analyses were repeated, comparing the pediatric strains to only the hpEurope and/or hpAfrica1 strains included in the publication by Gressmann et al. (25). Because the arrays include genes present in an hpEurope strain (26695) and an hpAfrica1 strain (J99), other hpEurope and hpAfrica1 strains have significantly fewer genes absent based on array CGH compared to other populations of *H. pylori* (25). To avoid bias in the comparison, the test for difference in mean percentages of genes absent was repeated, restricting the adult strains to only hpEurope and hpAfrica1 strains. The analysis of disproportionate distribution of genes variable only in adult strains among different functional classes was repeated, restricting the adult strains to only hpEurope strains, the group to which most of the North American pediatric strains likely belong (20, 25). Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

Microarray data accession number. Raw microarray data from this study are available in GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE13317.

RESULTS

A pediatric cohort of *H. pylori*-associated gastric symptoms. The patients' demographic characteristics, endoscopic presentation, and histological scores are presented in Table 1. Histology slides of biopsy material from the antrum were obtained from 35 of the 47 patients and from the fundus from 22 patients. There were 15 patients with biopsies available for both sites and 5 patients with no histology samples. All but five children had neutrophils and mononuclear cells in at least one of the gastric sites. Of these five children, two showed no inflammation, and one of these two had marked complete intestinal metaplasia. The remaining three children had only mononuclear cells corresponding to chronic gastritis. Of the 15 patients with biopsies from the antrum and the fundus, 12 had different quantitation of parameters for the two sites. Six had discordant scores for *H. pylori* colonization density (two had higher scores for antrum, and four had higher scores for fundus), seven had discordant scores for neutrophil infiltration (five had higher scores for antrum, and two had higher scores for fundus), and eight had discordant scores for monocyte infiltration (six had higher scores for antrum, and two had higher scores for fundus). While level of *H. pylori* density was positively correlated with level of neutrophil infiltration in this population ($P = 0.007$; Spearman's rank correlation), the discordant scores for inflammation between the two biopsy sites were not related to discordant *H. pylori* density scores.

RAPD typing shows evidence of mixed infection in a subset of patients. We performed RAPD typing of four single-colony isolates from each patient using two different primers to assess the relatedness of strains. As expected, clones from different patients yielded different RAPD patterns while clones from the same patient showed nearly identical DNA fragment pat-

TABLE 1. Patient characteristics

Parameter	Characteristic	Result[a]
Age (yr)		12 (3–21)[b]
Gender	Male	27 (57)
	Female	16 (34)
	Unknown	4 (9)
Race	White	24 (51)
	Black	15 (32)
	Other	5 (11)
	Unknown	3 (6)
Endoscopic presentation	Normal	22 (47)
	Nodularity	16 (34)
	Gastritis	2 (4)
	Ulcer	5 (11)
	Unknown	2 (4)
Antrum		
H. pylori density	Normal	2 (4)
	Mild	12 (26)
	Moderate	10 (21)
	Marked	11 (23)
	Unknown	12 (26)
Neutrophil infiltration	Normal	3 (6)
	Mild	7 (15)
	Moderate	13 (28)
	Marked	12 (26)
	Unknown	12 (26)
Monocyte infiltration	Normal	2 (4)
	Mild	3 (6)
	Moderate	5 (11)
	Marked	25 (53)
	Unknown	12 (26)
Fundus		
H. pylori density	Normal	4 (9)
	Mild	5 (11)
	Moderate	7 (15)
	Marked	6 (13)
	Unknown	25 (53)
Neutrophil infiltration	Normal	6 (13)
	Mild	3 (6)
	Moderate	8 (17)
	Marked	5 (11)
	Unknown	25 (53)
Monocyte infiltration	Normal	2 (4)
	Mild	5 (11)
	Moderate	8 (17)
	Marked	7 (15)
	Unknown	25 (53)

[a] Except as noted for patient age, values represent the number (percentage) of patients with the result ($n = 47$).
[b] Median and range.

terns regardless of the primer used (Fig. 1). In four patients, we observed one clone with a distinct RAPD pattern using both primers, suggesting infection with two distinct strain types. Two additional patients displayed differences with a single primer. Heterogeneous colony morphology observed in some patients did not correlate with observed differences in RAPD patterns. Thus, possibly six patients positive for *H. pylori* had infection with multiple strain types.

aCGH shows variability in genomic content. To further analyze the genetic differences among *H. pylori* isolates within and between patients we performed microarray-based CGH (aCGH) on one representative isolate of each RAPD type present in each patient. For one patient with apparently distinct clones by RAPD analysis, the second clone type did not grow well enough to obtain sufficient DNA for aCGH. We analyzed an additional clone obtained from a patient (Em38) (see below). Thus, we analyzed a total of 53 isolates from 47 patients.

We obtained data for 1,675 genes, of which 1,324 were present in all strains and 351 were absent in at least one of the strains (see Table S2 in the supplemental material). None of the genes was absent in all strains. Of the 351 genes that were variably present, 334 genes were also variably present among the 71 adult strains for which the results of aCGH were previously reported (25, 41) and 17 genes were variably present among only the pediatric strains. However, only seven of these genes (HP0105, HP0498, HP0843-0845, HP0888, and HP1265) have not been previously identified as variably present among *H. pylori* strains isolated from other populations (25, 29, 41, 42). HP0888 was absent in two strains and the other six genes were absent in only one strain. Of the 1,324 genes that were present in all of the pediatric strains, 197 were variably present among the adult strains. Two functional classes were overrepresented among the genes that were variably present in only the adult strains: transport and binding proteins ($P = 0.0006$; chi-square) and cell envelope proteins ($P < 0.0001$; chi-square). These two functional classes were also overrepresented when the analysis was restricted to the 15 hpEurope adult strains ($P = 0.003$ for transport and binding proteins and $P = 0.02$ for cell envelope proteins).

The percentage of genes absent for each pediatric strain ranged from 2.3% to 10.6%. In comparison, the percentage of genes absent in a global collection of 69 adult strains ranged from 6.6% to 17.7%. The mean percentage of genes absent from the pediatric strains (6.4%; standard deviation, 2.3%) was significantly lower than that of the 69 adult strains (10.7%; standard deviation, 2.4%) and the subset of 23 hpEurope and hpAfrica1 adult strains (9.4%; standard deviation, 1.8%), which may more accurately reflect strain variation representative of North America (20, 25) ($P < 0.0001$ for both comparisons; *t* test).

aCGH reveals variation in the presence of virulence genes. Among the variable genes were those whose presence has been associated with virulence, including the 27 *cag* PAI genes and the Lewis B antigen binding protein (*babA*) gene. Twenty-eight patients (60%) were infected with a strain having the *babA* gene. In addition, jhp0562, a gene associated with ulcer development in children but not adults (37), was variably present among the strains and 43 patients (81%) were infected with a strain having jhp0562. We were particularly interested in the *cag* PAI because its presence has been associated with severe disease in adults, but it was absent in the majority of strains in this population (Fig. 2). Interestingly, four of five patients with ulcer diagnosis contained a strain with a complete PAI versus

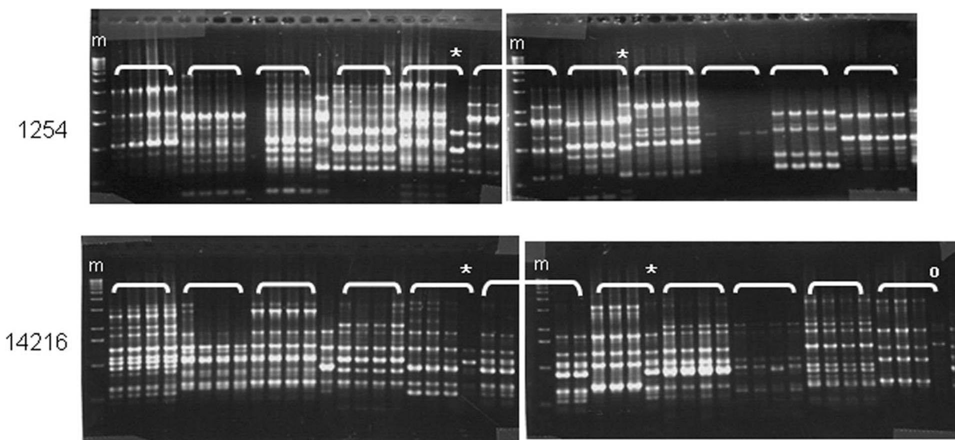

FIG. 1. Ethidium bromide-stained agarose gel showing RAPD profiles of four single-colony isolates per patient obtained with primers 1254 (top) and 14216 (bottom). Brackets identify clones from the same patient. m, marker; *, clone showing different RAPD patterns from the other three patient clones with both primers. °, clone showing a different RAPD pattern with a single primer (14216 in this example).

14 of 40 patients without ulcer. Since our initial RAPD typing analysis had revealed that infection with distinct strain types can occur in this patient population, we wondered whether the fifth ulcer patient (Em38) did indeed harbor a *cag* PAI-containing strain. To explore this possibility, we replated the initial frozen culture sweep, harvested 21 clones for analysis, and found 1 clone showing a distinct RAPD pattern (Em38-j14). Microarray analysis showed this clone contains all the genes in the *cag* PAI. Thus, in this cohort the *cagA* gene was present in 22 patients (47%), but only 19 (41%) bore a strain containing a complete PAI and therefore encoding a T4SS that could deliver CagA to host cells.

aCGH establishes coinfection with genetically distinct strains. Our RAPD analysis indicated seven patients might be infected with genetically distinct strains. We used the aCGH data to further explore the relatedness of clones from six of these patients (we were unable to obtain sufficient DNA from one clone). Pairwise comparison of gene content between clones showed that clones from the four patients with distinct RAPD patterns using both primers differed at 54 to 100 loci (Table 2). In contrast, the two patients with clones showing an altered RAPD pattern with only one primer differed at 13 and 15 loci (Table 2). Previous analyses of strains from children and adults revealed frequent infection with highly related clones that differ in 0 to 22 loci and rare cases in which much larger numbers of loci differ between clones that constitute distinct strains (42). Using these criteria, five of the seven patients with possible mixed infection had infection with distinct strain types, while two patients were infected with a single strain type. Thus at least 11% of patients had a mixed infection.

For two patients, the genes that varied between the distinct

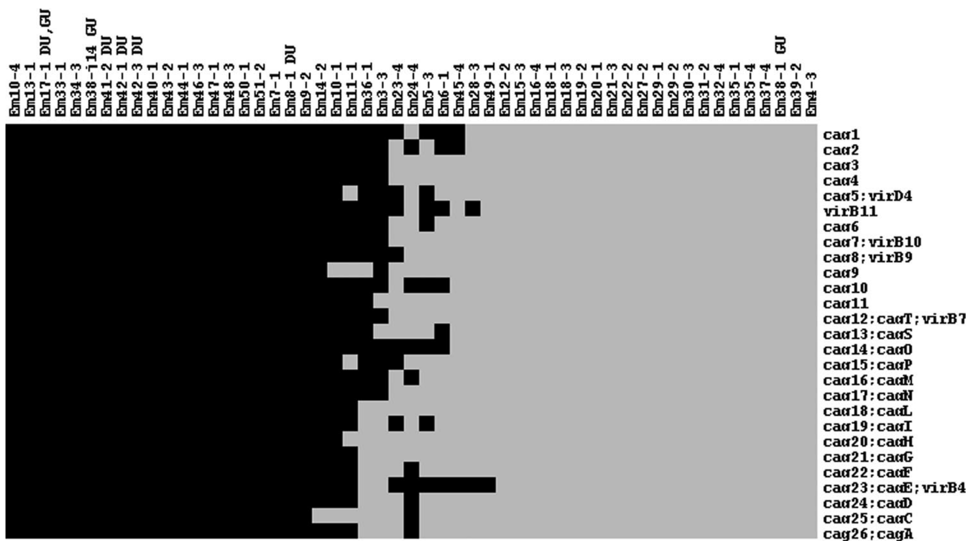

FIG. 2. Presence (black) or absence (gray) of the 27 *cag* PAI genes in 53 *H. pylori* clones isolated from 47 pediatric patients. Each column represents a different clone and each row the indicated gene. Clones isolated from patients with duodenal (DU) and gastric (GU) ulcers are indicated.

TABLE 2. Genetic content of strains from patients with two clones analyzed

Patient	Presence of RAPD differences[a]	No. of loci different[b]	*babA* status	*cag* PAI status
Em10	Both	92	10-1 yes, 10-4 no	10-1 partial, 10-4 full
Em18	Both	64	Both no	Both no
Em29	Only 14216	13	Both no	Both no
Em35	Only 1254	15	Both no	Both no
Em38	Both	100	Both yes	38-1 no, 38-j14 full
Em42	Both	54	Both yes	Both yes

[a] The RAPD primers were 1254 and 14216.
[b] The number of gene loci showing differential presence or absence between paired isolates as measured by aCGH.

infecting strain types may impart a different pathogenic potential (Table 2). In patient Em10, clone Em10-1 contained the *babA* gene but lacked *cag9* and *cag25*, while clone Em10-4 lacked *babA* but contained a complete *cag* PAI. In patient, Em38 both clones contained *babA*, but clone Em38-1 lacked the entire *cag* PAI, while clone Em38-j14 had a complete *cag* PAI. While the *cag* PAI has been shown to be unstable during human infection (30), clones Em38-1 and Em38-j14 differed at an additional 73 gene loci, making it unlikely that Em38-1 was a *cag* PAI$^-$ derivative of Em38-j14. In the remaining two patients containing distinct strain types, both either lacked (patient Em18) or contained (patient Em42) the full *cag* PAI and *babA*.

Analysis of bacterial virulence gene alleles. In addition to variation in presence and absence of virulence genes among strains, allelic variation of the secreted cytotoxins VacA and CagA correlate with disease severity in some populations. We used established PCR assays (8, 10) to analyze the VacA and CagA types present in this population. We determined the *vacA* allelic types at both the signal sequence region and the mid-region for all strains (see Table S1 in the supplemental material). Thirty patients (64%) were infected with a strain having the toxigenic *vacA* s1 allele, and 17 patients (36%) were exclusively infected with a strain having a *vacA* s2 allele. Of the 30 patients infected with a strain having a *vacA* s1 allele, 22 (73%) had the *vacA* s1/m1 allele, 5 (17%) had a *vacA* s1/m2 allele, 2 (7%) were positive for both the m1 and the m2 middle regions by PCR assay, and 1 (3%) had a band that was smaller than the m1 product by PCR assay.

For strains containing the *cagA* gene, we explored the number and type of CagA EPIYA phosphorylation site motifs present (Table 3). Among the 24 *cagA*-positive strains (from 22 patients), different CagA EPIYA motif configurations were observed. Seventeen (71%) had *cagA* alleles with the ABC EPIYA configuration, 1 (4%) had the BC EPIYA configuration, 2 (8%) had the BCC EPIYA configuration, and 1 (4%) had the ABCC EPIYA configuration. Three strains did not produce any product in the EPIYA PCR assay. However, one of the patients infected with a strain that did not produce any EPIYA PCR product had a mixed infection and the other strain had an ABC EPIYA motif configuration. Three of the patients infected with a strain having an ABC EPIYA motif configuration and one patient infected with a strain that did not produce any product in the EPIYA PCR assay did not have a full *cag* PAI.

Association of bacterial virulence genotypes with pathology. Of the 40 patients who did not have an ulcer, 14 (35%) were infected with a strain that had a complete *cag* PAI. In contrast, all five of the patients that had an ulcer were infected with a strain having a complete *cag* PAI. Infection with at least one strain containing a complete *cag* PAI was significantly associated with development of ulcer disease (by Fisher's exact test, $P = 0.0095$ when Em38-j14 [ulcer, PAI$^+$] is included and $P = 0.14$ when Em38-j14 is excluded). All 5 ulcer patients were also

TABLE 3. *cag* PAI genetic and phenotypic virulence characteristics of patient strains

Strain[a]	Presence of gene(s) by array[b]		*cagA* EPIYA[c]	Presence of CagA protein[d]	Translocation of CagA[e]		IL-8 release[f]	Ulcer[g]
	cag PAI	*cagA*			CagA delivery	Hummingbird phenotype		
Em44-1	Full	+	ABC	Yes	Yes	Yes	++	
Em10-4*	Full	+	ABC	Yes	No	No	−	
Em42-1*	Full	+	ABC	Yes	Yes	Yes	++	D
Em42-3*	Full	+	ABC	Yes	Yes	Yes	++	D
Em43-2	Full	+	ABC	Yes	Yes	Yes	++	
Em38-j14*	Full	+	ABCC	No	No	Yes	−	G
Em8-1	Full	+	BC	Yes	No	Yes	(+/−)	D
Em17-1	Full	+	BCC	Yes	Yes	Yes	+	G, D
Em41-2	Full	+	No bands	Yes	Yes	Yes	++	D
Em10-1*	Partial	+	No bands	No	No	No	−	
Em31-2	None	−		No	No	No	−	
Em38-1*	None	−		No	No	No	−	G
Em15-3	None	−		No	No	No	−	

[a] Asterisks indicate patients with two distinct strains.
[b] Gene presence assessed by aCGH.
[c] Measured by PCR typing.
[d] Assessed by immunoblotting.
[e] Translocation of CagA was determined by immunoblot detection of tyrosine-phosphorylated CagA or the hummingbird phenotype.
[f] IL-8 release was detected by ELISA during coculture of AGS cells with *H. pylori* strains.
[g] D, duodenal; G, gastric.

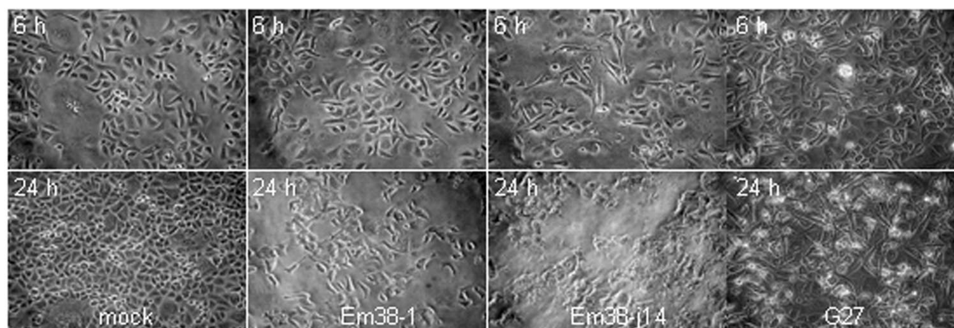

FIG. 3. Light micrograph of AGS cells cocultured with the indicated *H. pylori* strains for 6 h (top) or 24 h (bottom). Strain Em38-1 (PAI^-) does not display the hummingbird phenotype at either time point, and strain Em38-j14 (PAI^+) shows many elongated cells at 6 h and cytotoxicity at 24 h (compared to the mock and positive control strain G27 [PAI^+]).

infected with a strain having the *vacA* s1 allele and the jhp0562 gene, compared to 16 (40%) and 35 (88%), respectively, of the 40 patients who did not have an ulcer. Four (80%) of the 5 ulcer patients were infected with a strain having a *babA* gene, compared to 24 (60%) of the 40 patients who did not have an ulcer. However, the presence of neither the *vacA* s1 allele, the jhp0562 gene, nor the *babA* gene was significantly associated with ulcer disease (by Fisher's exact test, $P = 0.14$ for *vacA* s1, $P = 1$ for jhp0562, and $P = 0.64$ for *babA*). Of the 19 patients who were infected with at least one strain having a complete *cag* PAI, 16 were also infected with at least one strain having a *vacA* s1 allele and a *babA* gene. Having an infection that was positive for a complete *cag* PAI, a *vacA* s1 allele, and a *babA* gene was associated with having an ulcer ($P = 0.060$; Fisher's exact test). No significant associations were found between being infected with a strain having a complete *cag* PAI, a *vacA* s1 allele, or a *babA* gene and *H. pylori* colonization density, neutrophil infiltration, or monocyte infiltration.

***cag* PAI is functional in strains from patients with and without ulcer.** We saw a strong association for infection with a strain containing a complete set of *cag* PAI genes and development of ulcer disease. This led us to explore whether the T4SS system was functional in the strains from ulcer patients as well as within a subset of strains, including two with a complete set of *cag* PAI genes, one with a partial set, and two having none of the *cag* PAI genes, from patients without ulcer. To measure T4SS function, we measured translocation of the CagA effector into host cells and induction of proinflammatory cytokine release during coculture of each clinical isolate with a human gastric epithelial cell line (AGS) (Table 3; and see Fig. S1 in the supplemental material). To measure CagA translocation into host cells, we employed two assays. We used light microscopy to observe CagA-dependent host cell morphology changes, termed the "hummingbird" phenotype (45). We also monitored CagA entry into host cells by its acquisition of phosphorylation on tyrosine residues in the EPIYA motifs. As shown in Fig. S1A in the supplemental material, most but not all strains tested (6/8) containing a full complement of *cag* PAI genes induced IL-8 production above background, although there was considerable variation in the levels of IL-8 secreted in response to different isolates. All strains that induced IL-8 secretion induced the hummingbird phenotype, while strains lacking some or all of the *cag* PAI genes did not (Table 3; and see Fig. S1B in the supplemental material).

Interestingly, two strains (Em8-1 and Em38-j14) that induced little or no IL-8 secretion showed the hummingbird phenotype. Strain Em8-1 had detectable CagA protein, but no tyrosine-phosphorylated CagA could be detected even at 24 h (see Fig. S1C in the supplemental material). In the IL-8 assay, there was low, but detectable IL-8 secretion resulting in a +/− call (Table 3). These results suggest that the T4SS of this strain was in fact functional. Strain Em38-j14 showed no detectable IL-8 secretion in the coculture experiment, and neither CagA protein nor phospho-CagA could be detected with the antibodies used. Interestingly, light microscopy revealed a higher percentage of cells showing the hummingbird morphology at 6 h for strain Em38-j14 than for positive control strain G27 with a functional PAI (Fig. 3), suggesting an active *cag* PAI T4SS. At 24 h, when the G27 strain showed a high percentage of cells with the hummingbird morphology, cells incubated with Em38-j14 appeared to have lost membrane integrity and many detached from the substrate (Fig. 3). This cytotoxicity may account for the lack of detectable IL-8 production. In conclusion, the strains containing all *cag* PAI genes showed evidence by at least one of three assays that the T4SS and effectors were active in strains from 5/5 ulcer patients and 2/3 patients without ulcer. Conversely, 4/4 strains lacking all or part of the *cag* PAI showed no activity in any of the three assays.

DISCUSSION

This study investigated the genetic variability of *H. pylori* strains isolated from North American children. By comparing the genetic variability of the pediatric strains to what has been reported for adult strains, we observed that pediatric strains differ in their spectrum of strain-variable genes and the percentage of absent genes. Variability in the presence of virulence genes and virulence alleles was also observed among the pediatric strains. A relatively low proportion of the strains contained a complete *cag* PAI, but the presence of a complete *cag* PAI was associated with ulcer.

H. pylori strains show considerable genetic diversity between individuals, and individuals can be coincidentally infected with genetically distinct strain types (47). In order to correlate disease outcome with bacterial genotypes, it is necessary to assess all genotypes present in the infected person. Using RAPD followed by aCGH to quantify the number of loci different

among isolates, we found evidence that 11% of patients had infection with multiple genetically distinct strain types in a population with a low overall incidence of infection (14 to 15%) (Table 2). Previous work has suggested rare (0.8 to 7%) mixed infections among children mainly by observation of multiple *vacA* genotypes measured using PCR assays (17, 22, 39). Depending on the relative proportion of clones and the efficiency of PCR priming among different strains, the *vacA* PCR assay may underestimate the frequency of mixed infection. On the other hand, heterogeneity of *vacA* genotypes can be observed in an otherwise similar strain background (11), thus overestimating the frequency of mixed infection.

For one patient (Em38), analysis of 4 clones did not provide evidence of mixed infection, but screening of 25 clones revealed the presence of a strain with a distinct genotype from the rest of the population. Thus, our observation that 11% of patients harbor multiple strain types likely represents a minimal estimate of the true amount of mixed infection. In all cases of mixed infection, there appeared to be a dominant strain (75% or 96% of clones). While we were careful to allow sufficient time for growth of all clones during the initial plating and picked both large and small colony types when present, we cannot exclude the possibility that the observed strain prevalence was selected by in vitro growth.

We were motivated to search for an additional strain type in patient Em38 because of the gastric ulcer diagnosis and the absence of the *cag* PAI in the first strain isolated. Subsequent experiments confirmed the presence of an active *cag* PAI in the second strain type. If we hypothesize that this second strain was responsible for the pathological outcome, this suggests that a minority strain can still contribute to disease development. Alternatively, this clone's distribution may have been higher at the disease site (body) than the biopsy site (antrum) or selected against during growth in vitro.

The pediatric strains had a significantly lower percentage of genes absent compared to adult strains. It is possible that gene loss, including loss of genes that have a role in transmission and early infection, accumulates during the course of persistent infection. Because *H. pylori* infection is generally acquired in childhood, the pediatric strains may have a lower percentage of genes missing than adult strains because of their carriage for a shorter length of time from transmission. Strains that have a smaller amount of gene loss would be more likely to retain genes important for transmission and would be better able to infect a new host. Interestingly, while early molecular fingerprinting studies suggested that children acquire infection from their parents, and predominantly their mother, recent work has suggested that other household members contribute to transmission, including children (44). If recently transmitted strains in the pediatric population have a lower percentage of genes missing, they may represent a more robust source of new infections. Furthermore, examination of genes that are variably present in adult strains but always present in pediatric strains could help to identify genes that have a role in *H. pylori* transmission.

The genes that were lost in this pediatric population showed a different spectrum of functional annotation from that observed in adult isolate variable genes. Two functional classes, transport and binding proteins and cell envelope proteins, were overrepresented among the genes that were variably present in only the adult strains (and thus uniformly present in pediatric isolates). The cell envelope proteins that were variably present only in adult strains included many of the over 60 outer membrane proteins (OMPs) encoded in the *H. pylori* genome. Some OMPs have been shown to be adhesins (27, 32, 36). Because these proteins are expressed on the surface of the bacterial cell and interact with the host, having a larger repertoire of OMPs that can be expressed could be an advantage when *H. pylori* is adapting to a new host. Similarly, the *H. pylori* genome encodes many apparently redundant transport proteins, including multiple putative iron-siderophore uptake receptors (3). Whether these genes are truly redundant or transport a range of compounds found in different hosts remains to be determined. Certain *omp* or transporter genes that are important only during transmission and/or early infection or for which there is no receptor or substrate in a particular host may be more likely to be lost due to the absence of selection to maintain their presence during chronic infection. Interestingly, this gene loss is likely in just a subset of the strain population. Individual isolates from the same patient of the same strain differed in the carriage of 13 to 15 genes in this study (Table 2), and other studies using adults have observed up to 67 genes variably present within one person's strain population(28, 42). Possible mechanisms for this gene loss include the existence of direct sequence repeats in the genome that can mediate deletions (6) and observed gene conversion events between related outer membrane proteins (5, 46). The retention of a more complete spectrum of these cell envelope and transport and binding protein genes by pediatric strains suggests roles for these genes during transmission and/or early infection. Future studies that compare the genomic contents of pediatric strains and adult strains from the same population will help to further identify *H. pylori* genes involved in transmission. Additionally, pediatric strains may represent a better resource for studying the functional activity of these genes.

Using both microarray and PCR approaches, we were able to describe the frequency of adult bacterial virulence gene markers in our population. Interestingly, a complete and functional *cag* PAI showed the most significant association with ulcer disease development and addition of other adult bacterial virulence markers did not improve the association. Previous studies of pediatric populations using serology or PCR targeting CagA found an association in one case (40), but not others (17, 22), including North American children. Our phenotypic analysis showed that strains containing CagA, but lacking other genes of the PAI, do not induce proinflammatory and cell signaling phenotypes. On the other hand, most strains with a genetically complete PAI (8/9) displayed the expected cellular phenotypes and may better predict strain virulence. While one study suggested an association of CagA-positive strains with increased inflammation (34), we did not observe such an association. This may result from a lower control-to-case ratio when testing histologic parameters versus clinical diagnosis.

The prevalence of the *cag* PAI was lower (41%) than might be expected from the prevalence in North American adults (62%) predicted by CagA serology from a population-based survey(16). Other studies have observed a lower prevalence of CagA positivity in children compared to adults and an increase in CagA positivity with age (17, 40). Interestingly, in the two cases where mixed infection was observed with strains differing

in their *cag* PAI status, the *cag* PAI$^+$ strain appeared to be the minority strain. Since the immune responses of children to *H. pylori* infection appear to differ from those of adults(14, 23, 34), it is intriguing to speculate that a functional *cag* PAI may be detrimental to bacterial colonization or persistence in children while enhancing bacterial survival as the immune response matures. A better understanding of the interaction of *cag* PAI$^+$ strains with the pediatric immune system may provide clues as to why a complete *cag* PAI uniquely predicted development of pediatric ulcer disease compared to other adult *H. pylori* virulence gene markers.

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