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Virulence Genes and Neutral DNA Markers of *Helicobacter pylori* Isolates from Different Ethnic Communities of West Bengal, India

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Virulence-associated genes and neutral DNA markers of *Helicobacter pylori* strains from the Santhal and Oroan ethnic minorities of West Bengal, India, were studied. These people have traditionally been quite separate from other Indians and differ culturally, genetically, and linguistically from mainstream Bengalis, whose *H. pylori* strains have been characterized previously. *H. pylori* was found in each of 49 study participants, although none had peptic ulcer disease, and was cultured from 31 of them. All strains carried the *cag* pathogenicity island and potentially toxigenic *s1* alleles of vacuolating cytotoxin gene (*vacA*) and were resistant to at least 8 µg of metronidazole per ml. DNA sequence motifs in *vacA* mid-region m1 alleles, *cagA*, and an informative insertion or deletion motif next to *cagA* from these strains were similar to those of strains from ethnic Bengalis. Three mobile elements, IS605, IS607, and ISHp608, were present in 29, 19, and 10%, respectively, of Santhal and Oroan strains, which is similar to their prevalence in Bengali *H. pylori*. Thus, there is no evidence that the gene pools of *H. pylori* of these ethnic minorities differ from those of Bengalis from the same region. This relatedness of strains from persons of different ethnicities bears on our understanding of *H. pylori* transmission between communities and genome evolution.

Helicobacter pylori has been the focus of intense research efforts for some 20 years, motivated by recognition of its role as a major cause of peptic ulcer disease (46) and an early risk factor for gastric cancer (32), even though most infections are asymptomatic. It colonizes the gastric mucosa of more than half of all people worldwide (31). Infection tends to start in early childhood and to persist for years or decades once established (47). Much of the pathology associated with infection probably results from the host response rather than bacterial toxins or other virulence factors per se. The host response may be affected by bacterial genotype as well as by human host genotype and physiology and environmental conditions (6).

One of the most intriguing aspects of *H. pylori* is its great genetic diversity (1, 2, 13), which is at a level as yet unseen in other bacterial pathogens. Independent isolates generally differ from one another by some 3 to 5% base substitutions even in essential housekeeping genes, most of which, however, involve synonymous changes that do not affect amino acid sequence (20). Virulence-associated vacuolating cytotoxin gene (*vacA*) and *cagA* sequences are much more diverse, and much of this diversity reflects nonsynonymous base substitutions (1, 3, 12, 30). This pattern suggests selection for proteins with different structures, functions, or antigenic properties during different infections. The genetic diversity of *H. pylori* is postulated to reflect numerous factors: mutation (44), recombination between divergent lineages (42), selection based on differences among human hosts in traits that may be important to individual strains (14), and finally its non-epidemic (preferen-

tially intrafamilial) mode of transmission, which lowers the chance of selection for any one or a few possibly most fit genotypes (13, 29). Clinical isolates from adults are also likely to have diverged genetically from strains that may have infected them years before (in infancy), the result of years of selection for derivatives that are better adapted to the particular person, coupled with changes in gastric physiology over time. There are also differences in *H. pylori* genotypes that predominate in different geographic regions or human populations (16, 23, 28). This may reflect a combination of selection for different motifs (as in *vacA* and *cagA* above) plus genetic drift resulting simply from the highly localized mode of *H. pylori* transmission and isolation by distance of *H. pylori* strains in different human populations. Studies of *H. pylori* genotypes from different distinct human populations may contribute to our understanding of bacterium-host interactions and the origin and evolution of this bacterium in humans.

H. pylori infection is very common in India (28; H. H. Gill, P. Majumdar, K. Shankaran, R. H. Kalro, and H. G. Desai, Abstr. Annu. Conf. Ind. Soc. Gastroenterol., Ind. J. Gastroenterol. 9[Suppl. 1]:A17, 1990), and duodenal ulcer which is *H. pylori*-associated is of particular importance here and is far more common than in most other geographic regions (27). We had characterized the genotypes of *H. pylori* strains from adult ethnic Bengalis in Calcutta, in the state of West Bengal (8, 28). However, the Indian population is diverse, with close-knit communities, each consisting of peoples of different ethnicities, linguistic groups, and/or social status often living quite close to one another. Despite possibilities for transmission of *H. pylori* between unrelated people (e.g., through unclean water, food, or utensils), it was not clear a priori that the characteristics of mainstream Bengali *H. pylori* strains will fully

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represent the range of genotypes of *H. pylori* in other Indian communities, especially given ideas of how *H. pylori* tracks with human migration (11, 16, 23).

Santhals and Oroans are two distinct ethnic groups that had settled in the Birbhum district of West Bengal centuries ago (37–39). They constitute less than 5% of the overall population of West Bengal (Census of India, 2001). Ethnically, Santhals are proto-Australoids and speak the dialect Santhali of the Austro-Asiatic language family (37, 39), while the Oroans are ethnically Dravidian and speak the Khurukh dialect of the Dravidian linguistic family (38). That is, the two lineages to which they belong have been distinct for millennia. In contrast, the ethnic Bengalis have an Indo-European ancestry, and their Bengali language is derived from Sanskrit. Some scholars trace the migration and settlement of the Santhal communities in Bengal through the protohistoric and historic eras and link up their traditional homeland to Central India (initially known as the Dandakaranya area; in present-day Chattisgarh State). Later they came to the eastern flank of Dandakaranya (now in Bihar and Orissa States) and further migrated eastward to West Bengal (37). The original home of Oroans was in the western part of India, whence they came to the Kaimur hills and the plateau of Rohtas in Shahabad. Driven from Rohtas by Muhammadans, they followed the course of the Ganges and settled in Rajmahal hills and Chotonagpur plateau of Bihar, and from there migrated into adjacent West Bengal (38). Traditionally both Santhals and Oroans have been hunter-gatherers, but most have now become settled agriculturists, though they nevertheless continue to hunt and fish to supplement food. However, many of these peoples have begun to take other employment, typically as laborers in factories, mines, and farms of others (38). Nevertheless, they remain culturally, and linguistically distinct from most of Indian society and rarely intermarry with people of other ethnicities in India. Their separation from mainstream Bengalis and other Indians during much of human history is reflected in genetic differences in autosomal and mitochondrial DNA markers (7, 39).

These considerations and our interest in the population genetics and evolution of *H. pylori* motivated the present study of the prevalence and genotypes of strains from these two ethnic minorities in Bengal.

MATERIALS AND METHODS

Patient-derived samples. A total of 49 healthy adults of both sexes (age 19 to 40 years) of the Santhal [31] and Oraon [18] ethnic communities of West Bengal underwent nonsedated upper gastrointestinal endoscopies using a GIF XQ 30 endoscope (Olympus Optical Company, Tokyo, Japan) under topical lignocaine anesthesia at the Institute of Post Graduate Medical Education and Research, Calcutta. A detailed history was taken, and a physical examination of each subject was carried out prior to endoscopy. The objectives of the study were explained to all, and written informed consent was obtained from them under protocols approved by the ethical committee of the Institute of Post Graduate Medical Education and Research, Calcutta. The endoscope was carefully cleaned and disinfected between patients by first immersing the tube in detergent solution for 10 min and then rinsing it with sterile distilled water. During gastroscopy, three biopsy samples were taken from the antrum and body of the stomach for rapid urease test (RUT), histology, and culture. For RUT, one biopsy sample was inoculated into urea broth containing a pH-sensitive marker (phenol red), and any color change was noted within 10 to 30 min. A positive result was recorded if the color changed from yellow to pink within 30 min. Another biopsy sample was fixed in buffered 10% formalin for histopathological examinations, and the third was placed in 0.5 ml of brucella broth (Difco Laboratories, Detroit, Mich.) containing 15% glycerol and transported to the mi-

crobiology laboratory on ice, where it was inoculated immediately onto culture medium (below) or stored at -70°C .

Histopathological examination. Formalin-treated tissue biopsies were processed for paraffin embedding, followed by cutting thin (4- μm) sections, which were stained with modified Giemsa stain. Slides were examined microscopically using $\times 40$ magnification. If *H. pylori* was observed, the bacterial density was graded semiquantitatively on an ordinal scale (ranging from 0 to 3) based on the Sydney system (36) by a single pathologist. All examinations were performed under code. If the two biopsies from each site (antrum or corpus) showed a different grade, then the higher grade was recorded.

***H. pylori* culture.** Biopsy samples in transport medium were vortexed vigorously for 2 min, and 100 μl of the media was plated on brain heart infusion (BHI) agar (Difco) supplemented with 7% horse blood, 0.4% IsoVital-X, and *H. pylori* dent supplement (Oxoid, Basingstoke, Hampshire, England) (8). Plates were incubated at 37°C in an atmosphere of 5% O_2 to 10% CO_2 to 85% N_2 for 3 to 6 days. *H. pylori* bacteria were identified based on characteristic colony morphology and appearance on Gram staining, positive urease, and gene-specific PCR tests. *H. pylori* cells that grew out of one biopsy on the primary culture plate were collected as a pooled population and preserved in sterile BHI broth with 15% glycerol at -70°C . In general only one such culture was analyzed per individual.

Determination of antimicrobial susceptibility and resistance. Resistance or susceptibility to metronidazole (MTZ) (Sigma Chemical Co., St. Louis, Mo.) and clarithromycin (Sigma) were determined in this study. *H. pylori* strains growing exponentially on drug-free BHI agar were suspended in phosphate-buffered saline buffer, a series of 10-fold dilutions of these cell suspensions were prepared, and 10 μl of each dilution was spotted on freshly prepared BHI agar containing various concentrations of MTZ (0, 0.2, 0.5, 1.5, 3, 8, 16, 32, 64, and 128 $\mu\text{g/ml}$) (essentially as described in reference 22). The susceptibilities of strains to MTZ are described here in terms of MIC, defined operationally as the lowest of the MTZ concentrations listed above that reduces the efficiency of colony formation by at least 10-fold. We use this culture dilution protocol because it is more sensitive and reliable than traditional standard agar dilution or E-test methods for studying susceptibility to MTZ (MIC) with *H. pylori*, as discussed previously (23). For clarithromycin, the concentrations used ranged from 0.125 to 32 $\mu\text{g/ml}$. The plates were incubated for 3 to 4 days at 37°C under microaerophilic conditions. *Mtz*⁺ and *Clr*⁺ transformant derivatives of *H. pylori* reference strain 26695, clarithromycin-resistant strains 5883 and 5898 (45), and three *Mtz*^r Bengali *H. pylori* isolates (I-18, I-67, and I-86) (28) from our collection were included in each test as controls. For MTZ, resistance was defined as a MIC of $>8 \mu\text{g/ml}$, while for clarithromycin, resistance was defined as a MIC of $>2 \mu\text{g/ml}$ (45).

DNA methods. Chromosomal DNA was prepared by the hexadecyltrimethyl ammonium bromide extraction method from confluent BHI agar plate cultures. Specific PCR was carried out in 20- μl reaction volumes using 10 pmol of each primer per reaction, 0.25 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Takara, Shuzo, Tokyo, Japan), and 10 ng of DNA (from both the pool of colonies and single-colony isolates) in a standard PCR buffer (Takara). PCR was carried out for 30 cycles of the following: (i) denaturation at 94°C for 1 min, (ii) primer-template DNA annealing at 55°C for 1 min, and (iii) DNA synthesis at 72°C for a time based on expected fragment size (1 min/kb). The primers used are listed in Table 1. PCR products were purified with the QIA quick gel extraction kit (Qiagen Corporation, Chatsworth, Calif.) according to the manufacturer's instruction and were directly sequenced using the BigDye terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) on an automated DNA sequencer (ABI Prism 310). DNA sequence editing and analysis were performed with programs in the GCG package (Genetics Computer Group, Madison, Wis.), programs and data in The Institute for Genomic Research *H. pylori* genome database (<http://tigr.org/tdb/mdb/hpodb.html>), and the Phylip program of J. Felsenstein (<http://evolution.genetics.washington.edu/phylip.html>). Phylogenetic trees were visualized using Treeview (version 1.61 [<http://www.taxonometry.zoology.gla.ac.uk/rod/treeview.html>]).

Statistical analysis of data was performed by using the chi-square test and Fisher's exact test with significance set at a *P* value of <0.05 .

Nucleotide sequence accession numbers. The sequences obtained here were deposited in GenBank under the following accession numbers: AY162446 to AY162453 for 219 bp of *cagA* sequence and AY167579 to AY167584, AY168866 to AY168872, and AY169676 for 648 bp of *vacA* m1 alleles from Santhal and Oraon strains.

RESULTS

Incidence of *H. pylori*. All 49 persons of the two ethnic communities (31 Santhal, 18 Oraon) who participated in this

TABLE 1. Primers used in this study

Region	Primer	Nucleotide sequence	Reference
<i>vacA</i> s1 or <i>vacA</i> s2	VA1-F VA1-R	5'-ATG GAAATACAACAAACACAC 5'-CTGCTTGAATGCGCCAAAC	28
<i>vacA</i> m1b	Vam-F3 Vam-R3	5'-GGCCCCAATGCAGTCATGGAT 5'-GCTGTTAGTGCCTAAAGAAGCAT	28
<i>vacA</i> m2	VA4-F VA4-R	5'-GGAGCCCCAGGAAACATTG 5'-CATAACTAGCGCCTTGCAC	28
<i>vacA</i> 0.7-kb middle	VAm-F VAm-R	5'-GCTCATTACGGCTTCCACTAATGT 5'-GCGGTTATTGTTGTTATAAAGGGCTA	28
<i>cagA</i> (5' end)	cagA5 cag A2	5'-GGCAATGGTGGTCTGGAGCTAGGC 5'-GGAAATCTTTAATCTCAGTTCGG	28
<i>cag</i> -PAI empty site	Luni 1 R5280	5'-ACATTTTGGCTAAATAAACGCTG 5'-GGTTGCACGCATTTTCCCTTAATC	28
<i>iceA1</i>	iceA1F M.Hpy1R	5'-TATTTCTGGAAC TTGCGCAACCTGAT 5'-GGCCTACAACCGCATGGATAT	28
<i>iceA2</i>	cycSF IceA2R	5'-CGGCTGTAGGCACTAAAGCTA 5'-TCAATCCTATGTGAAACAATGATCGTT	28
<i>iceA1</i> Δ94 bp	A1F673 A1R1174	5'-GGTGAGTCGTTGGGTAAGCGTTACAGAATT 5'-CACAACCATCATATTACGCCTCCCCCTCATA	28
IS605 <i>orfA</i>	ORF18F ORF18R	5'-CGCCTTGATCGTTTCAGGATTAGC 5'-CAACCAACCGAAGCAAGCATAATC	28
IS605 <i>orfB</i>	ORF19F ORF19R	5'-GGCTGTTCTAGGGTCGTGTATAAC 5'-CAAGCTAGATGCAATCTAGCTACC	28
IS607	IS607 Flk.F IS607 F2	5'-GGCTACAAACAGAACTAAAAT 5'-AGATTACCGAATTTATAGATACG	25
IS608	608-F1 608-R	5'-CCATAACGCCTTAATAGTGTGTC 5'-CAAGCTTTGGAGTGATGAAGTTC	26
Type III	fcu unk cagF4856	5'-TGGATTAAATCTTAATGAATTATCG 5'-GCGATGAGAAGAATATCTTTAGCG	23

study were asymptomatic and appeared to have normal stomach and duodenal lining during visual examination during endoscopy. *H. pylori* was detected by RUT and histology in biopsies from every participant. Giemsa-stained tissue sections were carefully examined, and *H. pylori* colonization densities on gastric epithelia were graded on a four-point scale of none (grade 0), mild (grade 1), moderate (grade 2), or severe (grade 3), according to Sydney system guidelines (36). Of 49 samples, none belonged to grade 0, 13 (27%) were grade 1, 20 (41%) were grade 2, and 16 (33%) were grade 3. *H. pylori* was cultured from 20 of 31 biopsies from Santhals (64.5%) and 11 of 18 biopsies from Oroans (61%).

Genotypes of *H. pylori* strains. (i) *cag* PAI. The presence or absence of the *cag* pathogenicity island (PAI) was scored by PCR with specific primers using DNA extracted from cultured strains. A 324-bp product indicative of the *cag* PAI was obtained with primers specific for the *cagA* gene from each of the 31 strains. None yielded a 550-bp product expected of a *cag* empty site, which would indicate complete absence of the *cag* PAI (Table 2).

The 324-bp *cagA* PCR fragment is useful for assessing phylogenetic relationships (43) and was sequenced from eight representative strains (five Santhal, three Oroan). The eight sequences were closely related to one another and to corresponding *cagA* sequences of mainstream Bengali strains as well as those of ethnic European strains (28) and were distinct from those of East Asia (Fig. 1).

(ii) *vacA* alleles. The presence of potentially toxigenic *vacAs1* versus nontoxigenic *vacAs2* alleles at the 5' end of *vacA* was determined based on sizes of PCR products (259 versus 286 bp, respectively) generated with *vacAs* region-specific primers. All 31 Santhal and Oroan strains yielded a 259-bp

fragment, indicating that they carried s1 alleles; no s2 alleles were found (Table 2). The frequency of *vacA* s1 alleles observed here (100%) is higher ($P < 0.02$) than that seen for strains from mainstream Bengalis (8, 28).

The alleles of the *vacA* middle (m) region, which determines the cell type specificity of vacuolating cytotoxin action, were also studied by PCR. Products were obtained only with *vacA* m1b/c primers with 12 of 20 Santhal strains and 10 of 11 Oroan strains; only with *vacA* m2 primers with five Santhal strains and

TABLE 2. Genotypic characteristics of *H. pylori* from Santhal and Oroan communities

Trait or marker ^a	No. of strains with trait or marker/no. tested	
	Santhals (n = 20)	Oroans (n = 11)
<i>cag</i> PAI ⁺ only ^b	20/20	11/11
<i>cag</i> PAI ⁻ only ^b	0/20	0/11
<i>cag</i> Type III	13/20	11/11
<i>vacAs1</i> only	20/20	11/11
<i>vacAs2</i>	0/20	0/11
<i>vacAm1c</i>	12/20	10/11
<i>vacAm2</i> only	5/20	1/11
<i>vacAm1c</i> , -m2 mixed	3/20	0/11
<i>iceA1</i>	10/20	3/11
<i>iceA2</i>	9/20	4/11
<i>iceA1</i> , <i>iceA2</i> mixed	1/20	4/11
IS605	5/20	4/11
IS606	1/20	0/11
IS607	2/20	4/11
ISHp608	2/20	0/11

^a Distribution of DNA markers was determined by PCR.

^b *cag* PAI⁺ only, infection with strains carrying *cag* PAI only; *cag* PAI⁻ only, infection with strains lacking the *cag* PAI only.

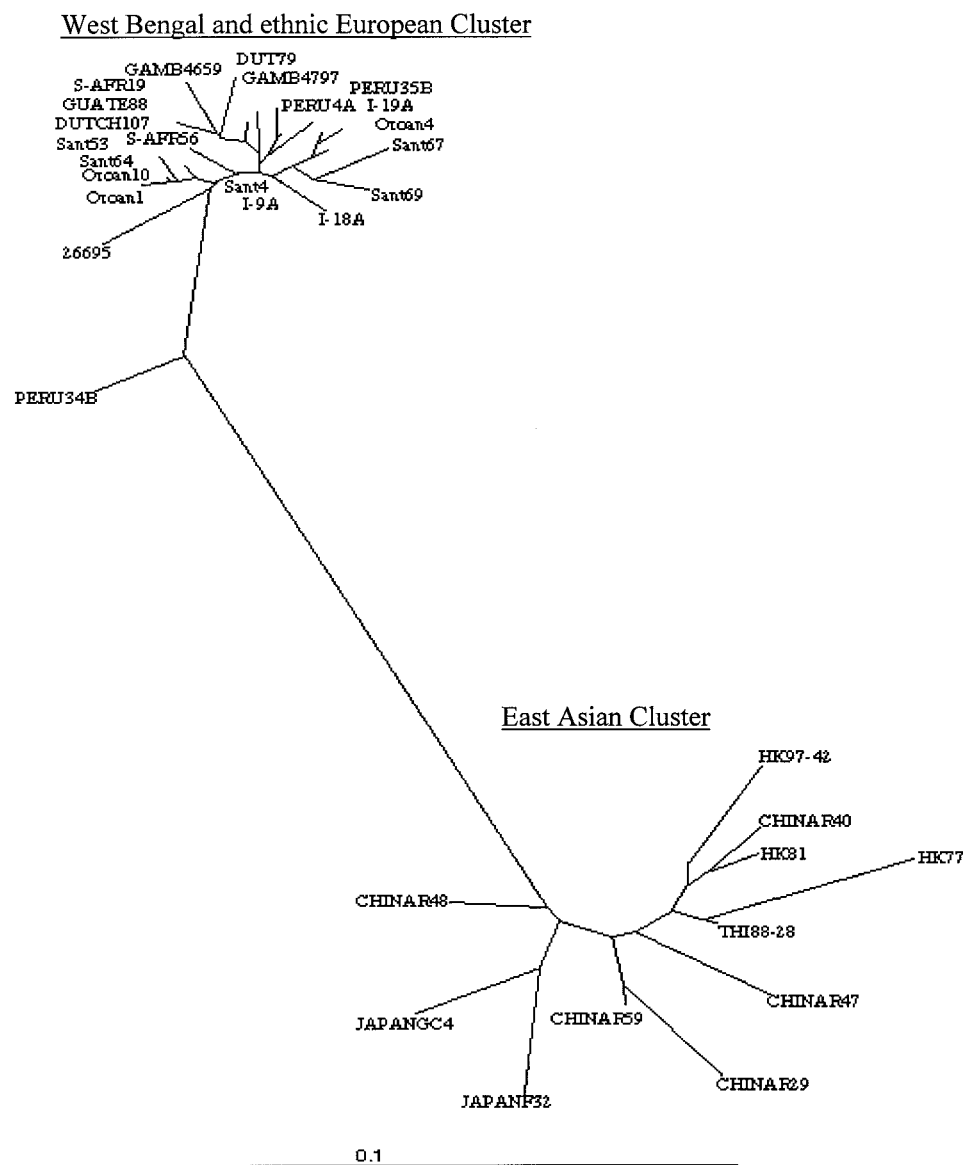


FIG. 1. Phylogenetic tree based on an informative 220-bp segment at the 5' end of *cagA* of *H. pylori* strains. The tree was generated using PHYLIP (Phylogeny Inference Package), version 3.5c, of J. Felsenstein. The strains used are as follows (GenBank accession numbers are in parentheses): 26695 (AE000569); I-18 (Calcutta, India) (AF202224); I-9 (Calcutta, India) (AF202225); Peru4A (AF198477), Peru34B (AF198475), Peru35B (AF198476), DUT79 (Dutch) (AJ252970), GAMB4659 (Gambia) (AF198468), GAMB4797 (Gambia) (AF198469), GUATE88 (Guatemala) (AF198472), S-AFR19 (South Africa) (AF198470), DUTCH107 (AJ252963), HK77 (Hong Kong) (AF198485), THI88 to 28 (Thailand) (AJ239722), HK97 to 42 (Hong Kong) (AJ239733), HK81 (Hong Kong) (AF198486), CHINAR47 (AJ252985), CHINAR40 (AJ252982), CHINAR59 (AJ252986), CHINAR29 (AJ252980), JAPANF32 (AJ239726), JAPANGC4 (AF198484), CHINAR48 (AJ252983), Sant 4 (Santhal) (AY162446), Sant 53 (Santhal) (AY162447), Sant 64 (Santhal) (AY162448), Sant 67 (Santhal) (AY162449), Sant 69 (Santhal) (AY162450), Oroan 1 (AY162451), Oroan 4 (AY162453), Oroan 10 (AY162452), and S-AFR56 (South Africa) (AF198471).

one Oroan strain; and with both m1b/c and m2 primers with three Santhal strains (indicating mixed infection; these cultures, although free of non-*H. pylori* bacteria, had not been purified by single-colony isolation before PCR analysis).

A 0.7-kb PCR fragment containing *vacA* mid-region alleles was sequenced from single-colony isolates of 10 Santhal strains and four Oroan strains that had yielded products with *vacA* m1b/c-specific primers. Analyses of sequences revealed that all were closely related to one another and to the *vacA* m1c alleles that are characteristic of mainstream Bengali *H. pylori* strains

(28) and that each was distinct from European m1a alleles and East Asian m1b alleles (Fig. 2).

(iii) *iceA* alleles. PCR was used to test for *iceA1*, which is virulence associated in some populations, and the completely unrelated *iceA2* gene, which occupies the same chromosomal locus in strains lacking *iceA1* (34). The *iceA1* gene was found alone in 10 of 20 Santhal cultures and in 3 of 11 Oroan cultures, *iceA2* was found alone in nine Santhal cultures and four Oroan cultures, and a mixture of *iceA1* and *iceA2* alleles (again, indicating mixed infection) was found in one Santhal

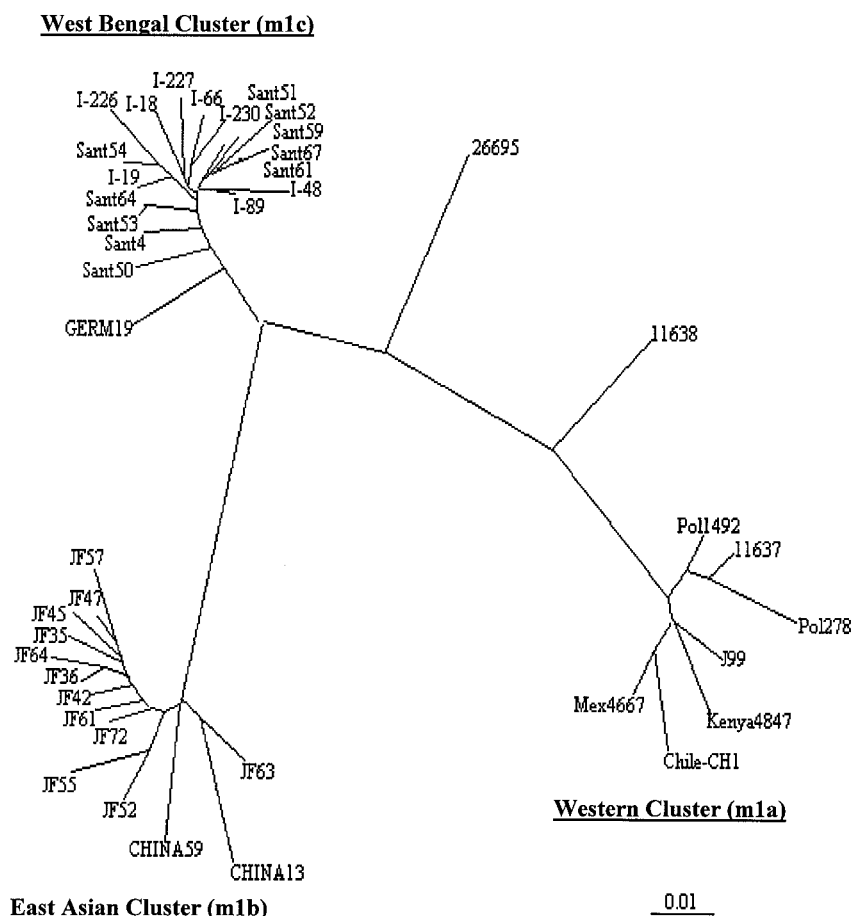


FIG. 2. Phylogenetic tree based on an informative 650-bp segment of *vacA* gene containing *vacAm1* alleles of *H. pylori* strains. The tree was generated using PHYLIP (Phylogeny Inference Package), version 3.5c, of J. Felsenstein. The strains used are as follows (GenBank accession numbers are in parentheses): I-19 (Calcutta, India) (AF220111), I-226 (Calcutta, India) (AF220115), I-89 (Calcutta, India) (AF220114), I-48 (Calcutta, India) (AF220112), I-18 (Calcutta, India) (AF220110), I-230 (Calcutta, India) (AF220117), GERM19 (Germany) (AJ006967), I-66 (Calcutta, India) (AF220113), I-227 (Calcutta, India) (AF220116), JF52 (Japan) (AF049631), JF55 (Japan) (AF049632), CHINAR59 (AF035611), JF63 (Japan) (AF049635), CHINAR13 (AF035610), JF42 (Japan) (AF049626), JF72 (Japan) (AF049651), JF45 (Japan) (AF049628), JF47 (Japan) (AF049629), JF57 (Japan) (AF049634), JF35 (Japan) (AF049625), JF61 (Japan) (AF049645), JF36 (Japan) (AF049462), JF64 (Japan) (AF049647), JF45 (Japan) (AF0496628), Pol1492 (Poland) (AF097570), Pol278 (Poland) (AF097571), NCTC11637 (AF049653), J99 (AE001511), NCTC11638 (U07145), 26695 (AE000598), Kenya AFN4847 (AF191644), Chile-CH1 (AF479031), and Mex 4467 9 (Mexico) (AF159855).

culture and four Oroan cultures (Table 2). None of the 18 *iceA1* alleles found here contained the 94-bp deletion near the 3' end of *iceA1*, which was found in about one-fifth of mainstream Bengali strains (28).

(iv) **cag Right junction motifs.** The type III motif at the extreme right end of *cag* PAL, which predominates in *H. pylori* strains from mainstream Bengali strains (8, 23, 28), was found by PCR with diagnostic primers in 13 of 20 Santhal strains and in all 11 Oroan strains.

(v) **Neutral markers.** PCR tests for insertion sequences, which may be considered neutral markers, identified IS605 in 5 of 20 Santhal strains and 4 of 11 Oroan strains, IS606 in just one Santhal strain and no Oroan strains, IS607 in two Santhal strains and four Oroan strains, and ISHp608 in two Santhal strains and no Oroan strains. Only 1 of the 31 cultures contained two IS elements (IS605 and IS607), and 14 lacked each of these elements (Table 2). Thus, each element may be carried independently of the others, and the prevalence is similar to that for Bengali *H. pylori* (25, 26, 28).

Antimicrobial susceptibility. All Santhal and Oroan *H. pylori* strains were sensitive to even 0.125 μ g of clarithromycin/ml, as is typical of mainstream Bengali *H. pylori* strains (28), whereas known resistant control strains 5883 and 5898 (45), tested in parallel, were scored as resistant to 8 μ g of clarithromycin per ml, as expected. In contrast, all strains were resistant to MTZ, with MICs of 16 μ g/ml (18 strains), 32 μ g/ml (10 strains), or 64 μ g/ml (3 strains). Control strain 26695 tested in parallel was scored as sensitive to MTZ (MIC of 4 μ g/ml). An extremely high prevalence of MTZ resistance (>90% of strains) is also typical of mainstream Bengali strains (9, 28).

DISCUSSION

Here we report that *H. pylori* infection is common in the Santhal and Oroan ethnic minorities of West Bengal, if anything even more common than in mainstream Bengali society, whereas overt *H. pylori*-associated disease is rare in these people, even though the genotypes of the strains they carry are

similar to those for mainstream Bengalis. The near-universality of *H. pylori* infection can be ascribed to relatively low levels of sanitation, hygiene, and education (37), conditions that contribute to a high risk of infection and superinfection, even in adulthood (21, 41). A continuing risk of infection throughout life may explain occasional findings of mixed infection even in analyses of strains from single biopsies. Such results probably underestimate true frequencies of mixed infection for the following reasons: (i) *H. pylori* infections are often patchy (4), such that any single biopsy may often contain only a subset of strains that may be carried by a mixedly infected person, and (ii) mixed infections were detected here by PCR for conserved markers and insertion/deletion motifs, not point mutation differences, which are the most common polymorphisms in *H. pylori* populations.

The indication that Santhal and Oraon strains are closely matched to those of mainstream Bengalis is based most critically on the intermingling of phylogenetic trees of sequences from *cagA* and *vacA* genes from the strains obtained from these three communities and PCR data indicating similar frequencies of various markers, in particular, the four IS elements, *iceA1* versus *iceA2* genes, and polymorphic sequences downstream of *cagA* (8, 23, 28). Since these ethnic minorities and other Bengalis are of very different ancestries, the observed strain similarities suggest transmission among the populations, presumably from the far more numerous mainstream Bengali population to these ethnic minorities. Transmission between communities might have (i) involved displacement of a preexisting distinct strain population (assuming that *H. pylori* had been endemic in these peoples before extensive contact with Bengalis, as some would predict (5, 11, 16), due simply to the far great abundance of Bengalis and thus of their strains in water supplies and other possible environmental sources or due to a possibly greater vigor of Bengali strains; or (ii) resulted from easy transmission to and epidemic spread within a highly susceptible population if the hunter-gatherer ancestors of these ethnic minorities were *H. pylori* free, as predicted by a model in which *H. pylori* infection became widespread in humans only after the start of agriculture (23). It is striking that each of the 31 strains tested was MTZ resistant, since MTZ is used far less in Santhal and Oraon communities than in mainstream Bengali society (where some 90% of strains are also MTZ resistant) (9). Since MTZ only became widely available in India in the 1970s (9), this suggests that much of the postulated transmission from Bengalis to ethnic minorities may have been quite recent.

The carriage of the *cag* PAI and of putatively toxigenic *s1* alleles of *vacA*, which contribute to virulence when active, is in accord with the high abundance (about 90%) of this strain type in mainstream Indian populations (8, 28), the high overall risk of *H. pylori* infection in these populations, and the idea that frequent transmission tends to select for more virulent strains of a pathogen (15). This abundance is also remarkable, in light of the apparent absence of *H. pylori*-associated gastrointestinal disease in these populations. This suggests that on average, *H. pylori* infections are less virulent in these ethnic minorities than in mainstream Indians. Such avirulence might be due to subtle features of bacterial strains or aspects of the human host environment. For example, lesser virulence of the *H. pylori* strains themselves might be ascribed to subtle (e.g., point) mutations

that affect levels of expression of virulence genes or the potencies of their products, which do not, however, affect outcomes of diagnostic PCR tests used here. At least two reports of decreased virulence apparently being selected in vivo have appeared: one during human infection (24) and another during adaptation of human strains to mice (35). Avirulence might also reflect features of the host. One possibility entails concurrent infection with particular parasites that may downregulate inflammatory responses to infection, as has been documented in a mouse infection model (19). Resistance to pathogenic effects of putatively virulent *H. pylori* strains might also be determined by features of human host genotype. For example, studies using different inbred mouse lines have shown that the severity of inflammation, and thus potentially the risk of overt disease, that a given *H. pylori* strain can elicit is affected by the host genotype (17, 18). Indeed, the historic importance of cholera and other diarrheal diseases as a major cause of death in the Indian subcontinent and the importance of *H. pylori* infection in increasing susceptibility to enteric pathogens (10, 33, 40) might have helped select for human genotypes that result in decreased virulence during chronic infection.

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