

Population Genetic Analysis of *Helicobacter pylori* by Multilocus Enzyme Electrophoresis: Extensive Allelic Diversity and Recombinational Population Structure

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Genetic diversity and relationships in 74 *Helicobacter pylori* isolates recovered from patients assigned to distinct clinical categories were estimated by examination of allelic variation in six genes encoding metabolic housekeeping enzymes by multilocus enzyme electrophoresis. Seventy-three distinct allele profiles, representing multilocus chromosomal genotypes, were identified. All six loci were highly polymorphic, with an average of 11.2 alleles per locus. The mean genetic diversity in the sample was 0.735, a value that exceeds the level of diversity recorded in virtually all bacterial species studied by multilocus enzyme electrophoresis. A high frequency of occurrence of null alleles (lack of enzyme activity) was identified and warrants further investigation at the molecular level. Lack of linkage disequilibrium (nonrandom association of alleles over loci) indicates that horizontal transfer and recombination of metabolic enzyme genes have contributed to the generation of chromosomal diversity in *H. pylori*. In this sample of isolates, there was no statistically significant association of multilocus enzyme electrophoretic types or cluster of related chromosomal types and disease category.

The bacterium *Helicobacter pylori* participates in the pathogenesis of gastritis and peptic ulcer disease (4, 24). This pathogen has also been implicated in the etiology of gastric malignancies, including adenocarcinoma and lymphoma of mucosa-associated lymphoid tissue type (3, 15, 19, 38, 44). *H. pylori* is distributed globally (32) and may be the most common chronic infection of humans. Much research effort has been expended to delineate the molecular mechanisms of host-parasite interaction and specifically to identify bacterial products responsible for alteration in gastric structure and function. In addition, several groups of investigators have begun *H. pylori* genome projects that will ultimately result in considerable knowledge about the linear nucleotide sequence of one or more arbitrarily chosen strains (7, 57).

Despite the intense interest in the pathogen, relatively little is known about the population genetics of isolates classified as *H. pylori*. Data obtained by biotyping (28), plasmid profiling (42, 55), restriction fragment length polymorphism analysis of genomic DNA (1, 26, 30, 42, 43, 47, 49, 58) and specific loci (2, 11, 13, 14, 18, 20, 22, 48, 59), and repetitive extragenic palindromic PCR (REP-PCR) (23) have shown that there are substantial levels of variation among natural isolates of this pathogen. However, several important questions remain unanswered about the diversity observed among *H. pylori* isolates. For example, it is unclear if the diverse nature of the organism is due to the occurrence of a large array of clonal lineages that are evolving essentially in isolation from one another or, alternatively, if substantial levels of horizontal gene transfer and recombination are contributing to the generation of natural population diversity. The observations that some isolates of *H. pylori* are naturally competent (37, 62), many isolates contain

plasmids (42, 55), and some have phages (25, 50), together with reports of mosaic gene structures (2, 14, 18) suggest that recombination processes may be contributing to the observed overall species diversity. A second important unanswered question is the level of association, if any, of specific *H. pylori* strains with distinct clinical conditions. This is an especially noteworthy question because of the range of pathology associated with infection by this pathogen.

Multilocus enzyme electrophoresis is a convenient method to estimate levels of allelic variation in natural populations of bacteria (52, 54). In addition, through use of appropriate statistical techniques, the data can be used to infer the level of chromosomal horizontal gene transfer occurring in natural populations. This technique has also been useful for elucidating previously unrecognized nonrandom associations of intraspecies phylogenetic lineages with discrete infection types, host species, or other natural history correlates (54). The goal of the present study was to employ multilocus enzyme electrophoresis to index the level of metabolic enzyme gene allelic variation and probe the question of clinical syndrome-strain associations.

MATERIALS AND METHODS

Patient demographics and bacterial isolates. *H. pylori* isolates were recovered from tissue taken at biopsy from 74 individuals living in the Houston, Texas area. The sample includes 30 patients diagnosed with uncomplicated gastritis, 37 patients with a diagnosis of duodenal ulcers, and 7 individuals with gastric ulcers. The patients included 34 blacks, 28 Caucasians, 11 Hispanics, and 1 Asian, and there were 57 men and 17 women. The age range was 19 to 74 years, with a median age of 43 years. This sample of strains was chosen for analysis because of the need to have an unambiguous clinical history. Knowledge of this information was critical to study design and data interpretation. All biopsies were taken by one gastroenterologist, and all patients were diagnosed by one treating gastroenterologist.

Gastric biopsy samples for culture were obtained as part of a study protocol approved by the Baylor College of Medicine Human Subjects Institutional Review Board. Each individual who consented to undergo esophagogastroduodenoscopy had a single antral biopsy specimen obtained for culture. The biopsy material was streaked onto brain heart infusion agar supplemented with 7% fresh horse blood. Isolates with typical colony morphology, Gram stain spiral

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morphology, and biochemical tests positive for urease, catalase, and oxidase production were frozen. These are the standard tests used for species-level identification of *H. pylori*. Twenty-five of the organisms we studied have previously been characterized by DNA-DNA reassociation analysis and shown to have greater than 70% hybridization values (64). The sample of 25 organisms previously characterized represents the breadth of genomic diversity present among strains in that analysis and also in the present one.

Growth of bacteria and preparation of cell lysates. *H. pylori* grown from mucosal biopsy material was plated on brain heart infusion agar plates supplemented with 7% horse blood and incubated at 37°C in 12% CO₂. Bacteria growing in confluent cultures were then used to inoculate four 250-ml samples of Oxoid Iso-Sensitest broth containing 10% heat-inactivated horse serum. The bacteria were incubated for 2 to 3 days at 37°C in 12% CO₂ and harvested by centrifugation at 10,000 × g for 15 min. At harvest, all cultures were composed of organisms with typical spiral or rod morphology and were positive for urease, catalase, and oxidase activity.

The supernatant was discarded, and the bacterial cell pellet was suspended in 1.5 ml of 50 mM Tris-HCl-5 mM EDTA, pH 8.0. The cells were lysed by sonication with a model 200 Sonifier Cell Disrupter (Branson Sonic Power Co., Danbury, Conn.) equipped with a microtip for 30 s at 50% pulse with dry ice-methanol cooling. The sonicate was centrifuged at 20,000 × g for 10 min, and the supernatant was immediately used for horizontal starch gel electrophoresis (52). National Culture Type Collection strain *H. pylori* NCTC 11638 was used as a reference strain when necessary.

Multilocus enzyme electrophoresis and statistical analyses. Lysates were electrophoresed on starch gels and selectively stained for metabolic enzyme activity by methods of Selander et al. (52). The enzymes studied were glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase, indophenol oxidase, nucleoside phosphorylase, and adenylate kinase. Glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, glutamate dehydrogenase, and indophenol oxidase were electrophoresed in a Tris-citrate buffer (pH 8.0). A phosphate buffer system (pH 7.0 gel buffer and pH 6.7 tray buffer) was used for electrophoresis of nucleoside phosphorylase and adenylate kinase.

In addition to the six enzymes listed above, the following enzyme activities were either lacking in the bacterial lysates under the conditions assayed or not consistently scorable by the electrophoresis conditions used: aconitase, galactose-6-phosphate dehydrogenase, alanine dehydrogenase, threonine dehydrogenase, shikimate dehydrogenase, lactate dehydrogenase, phosphoglucosylase, glyceraldehyde-3-phosphate dehydrogenase, hydroxybutyrate dehydrogenase, 6-phosphogluconate dehydrogenase, leucine aminopeptidase, alcohol dehydrogenase, phosphoglucose isomerase, esterase, malate dehydrogenase, malic enzyme, leucylalanine peptidase, and glycylglycylglycine peptidase. For these enzymes, a search was made to detect activity by electrophoresis and staining for all enzymes on several different buffer systems, including Tris-citrate buffer (pH 8.0), a phosphate buffer system (pH 7.0 gel buffer and pH 6.7 tray buffer), a Poulik buffer system (pH 8.7 gel buffer and pH 8.2 borate tray buffer), Tris-maleate buffer (pH 7.4), Tris-maleate buffer (pH 8.0), a Tris-borate-EDTA buffer system (pH 8.0), and a Tris-citrate buffer system (pH 6.7 gel and pH 6.3 tray buffer).

Distinctive electromorphs (mobility variants) of each enzyme were numbered in order of decreasing rate of anodal migration and were equated with alleles at the corresponding structural gene locus. Equivalence in enzyme mobility was confirmed with side-by-side electrophoretic comparison. Each isolate was characterized by its combination of alleles at the six enzyme loci, and distinctive profiles of electromorphs, corresponding to unique multilocus enzyme genotypes, were designated electrophoretic types (ETs). Although many studies of the population structure of pathogenic bacteria have been conducted by indexing variation at a larger number of loci, use of only six loci usually does not substantially affect the primary conclusions.

Genetic diversity (*h*) at a locus was calculated as described previously (52). The mean genetic diversity per locus (*H*) was calculated as the arithmetic average of *h* values for all loci (52). The index of association between loci (*I_A*) was calculated as described previously by Maynard Smith et al. (31). The *I_A* value is a measure of the degree of association between loci and has an expected value of zero for large random mating (sometimes referred to as panmictic) bacterial populations. In contrast, for bacterial species that are clonal at all levels as a result of geographic isolation or infrequent genetic recombination, the expected value of *I_A* should be significantly different than zero.

RESULTS

Identification of a high frequency of occurrence of null alleles. Although 62% of the 75 strains had activity for all six enzymes, at least one metabolic enzyme activity could not be detected in the cell lysates prepared from 28 (37.8%) of the organisms. For example, 16 of these 28 isolates lacked detectable activity for glucose-6-phosphate dehydrogenase, and adenylate kinase activity was not found in lysates of 7 of 28

TABLE 1. Allele frequencies and genetic diversity at six enzyme loci in 73 ETs of *H. pylori*

| Enzyme locus ^a | No. of alleles | Genetic diversity (<i>h</i>) |
|---------------------------|----------------|--------------------------------|
| GLD | 12 | 0.849 |
| IPO | 6 | 0.549 |
| G6P | 12 | 0.866 |
| IDH | 9 | 0.521 |
| NSP | 12 | 0.710 |
| ADK | 16 | 0.913 |
| Mean | 11.2 | 0.735 |

^a GLD, glutamate dehydrogenase; IPO, indophenol oxidase; G6P, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; NSP, nucleoside phosphorylase; ADK, adenylate kinase.

isolates. Moreover, four isolates lacked detectable activity for two enzymes.

Two lines of evidence were generated in support of the idea that lack of enzymatic activity was due to the true absence of the enzyme, rather than a laboratory artifact associated with variation in the quality of the cell lysate. First, among organisms with null alleles, visual inspection of the gels histochemically stained for the other enzymatic activities assayed failed to reveal uniformly low activity. This result would be the expected outcome if lysates had been prepared in an inadvertently poor, sporadic fashion. Second, preparation of concentrated cell extracts from several isolates with apparent null alleles repeatedly grown on separate occasions to a high cell density also did not yield detectable activity for the target enzyme.

There was no apparent relationship between the presence or absence of detectable enzyme activity and disease category (e.g., uncomplicated gastritis, gastric ulcer, or duodenal ulcer).

Overall genetic diversity. In the collection of 75 isolates as a whole, all six loci were highly polymorphic. The mean number of alleles per locus was 11.2, and the number of alleles per locus ranged from 6 for indophenol oxidase to 16 for adenylate kinase (Table 1). Seventy-three distinct allele profiles, marking multilocus enzyme ETs were identified. The mean genetic diversity in the sample was 0.735, a value that exceeds the level of diversity recorded for other bacterial species studied by multilocus enzyme electrophoresis (Table 2).

When stratified by disease category, there was no significant difference noted in mean genetic diversity among isolates recovered from individuals with gastritis, gastric ulcer, or duodenal ulcer (Table 3).

Lack of effect of laboratory passage on electromorph profile. Growth of *H. pylori* NCTC 11638 at several independent times did not affect the electromorph mobilities of any of the six enzymes assayed (data not shown).

Index of association value. The frequency of recombination in natural populations can be estimated by calculating the index of association value between loci (31). The value calculated for the 75 isolates studied was 0.21 ± 0.16 and did not differ significantly from zero. Moreover, calculation of *I_A* for the 73 ETs resulted in a value of 0.20 ± 0.16 and did not differ significantly from zero. These results are consistent with the so-called panmictic model described by Maynard Smith et al. (31).

DISCUSSION

Recombinatorial structure of populations. Our findings are consistent with a relative lack of linkage disequilibrium in *H.*

TABLE 2. Genetic diversity among ETs in representative species of pathogenic bacteria

| Taxon | No. of ETs | Average no. of alleles per locus | Mean diversity (<i>H</i>) |
|--|------------|----------------------------------|-----------------------------|
| <i>Bordetella</i> spp. ^a | 14 | 2.4 | 0.284 |
| <i>Borrelia</i> spp. ^b | 35 | 5.9 | 0.673 |
| <i>Erysipelothrix</i> spp. ^c | 50 | 3.7 | 0.314 |
| <i>Haemophilus influenzae</i> ^d | 280 | 6.4 | 0.467 |
| <i>Helicobacter pylori</i> | 73 | 11.2 | 0.735 |
| <i>Legionella pneumophila</i> ^e | 50 | 3.2 | 0.312 |
| <i>Listeria monocytogenes</i> ^f | 45 | 3.6 | 0.424 |
| <i>Mycobacterium</i> spp. ^g | 33 | NA ^o | 0.580 |
| <i>Neisseria meningitidis</i> | | | |
| 8 primary serogroups ^h | 331 | 7.2 | 0.547 |
| Asymptomatic carriers ⁱ | 78 | 6.0 | 0.538 |
| <i>Neisseria gonorrhoeae</i> ^j | 89 | 2.5 | 0.410 |
| <i>Salmonella</i> spp. ^k | 80 | NA | 0.627 |
| <i>Staphylococcus aureus</i> ^l | 252 | 6.7 | 0.289 |
| <i>Streptococcus pneumoniae</i> ^m | 53 | 3.9 | 0.413 |
| <i>Streptococcus pyogenes</i> ⁿ | 33 | 4.4 | 0.420 |

^a Isolates of *B. pertussis*, *B. paraptussis*, and *B. bronchiseptica* (34).

^b Isolates of at least four *Borrelia* spp. (*B. burgdorferi*, *B. garinii*, *B. afzelii*, and *B. japonica*) (5).

^c Isolates of *E. rhusiopathiae* and *E. tonsillarum* (10).

^d Isolates of six capsule serotypes from global sources (35).

^e Isolates from intercontinental sources (53).

^f Isolates from endemic and epidemic cases in the United States and Europe (46).

^g Isolates of *M. avium* complex, *M. paratuberculosis*, *M. scrofulaceum*, *M. kansasii*, *M. chelonae*, *M. goodii*, *M. xenopi*, *M. bovis*, *M. tuberculosis*, *M. smegmatis*, and *M. fortuitum* (63).

^h Isolates from intercontinental sources (9).

ⁱ Isolates from individuals living in Norway (8).

^j Isolates from the United Kingdom (41).

^k Representative isolates of five *Salmonella* subspecies (51).

^l Isolates from humans with many distinct diseases and from cows and sheep with mastitis (36).

^m Carrier and invasive disease isolates from Finland (56).

ⁿ Invasive disease episodes in patients from throughout the United States (33).

^o NA, not available.

pylori and strongly suggest that horizontal gene transfer and recombination processes involving chromosomal genes participate in generating genomic diversity in natural populations of this pathogen. A recombinatorial population structure or other evidence for significant levels of horizontal chromosomal gene flow has been reported for only a few pathogenic bacterial species or subgroups of species. The organisms include *Neisseria gonorrhoeae* (40, 41), *Neisseria meningitidis* (16, 31, 61), and *Streptococcus pneumoniae* (12, 29), which like *H. pylori*, are naturally transformable.

In theory, a recombinatorial population structure could arise by processes involving transformation, transduction, or conjugation, or a combination of these. Because most isolates of *H. pylori* are naturally competent (62), we favor the idea that transformation is the main driving force. Transformation in natural populations could occur by uptake of DNA released by other *H. pylori* or by other organisms residing in the same milieu. Several investigators have reported that two or more distinct strains can exist in the stomach of a single individual, either at one time or over the course of months or years (22, 26, 58). Hence, the organism has both the molecular machinery and the ecological setup for strain-to-strain spread of DNA to occur. It will clearly be important to study the potential importance of horizontal gene transfer in scenarios such as therapeutic treatment failures, drug resistance, mosaic gene structure, generation of antigenic diversity, persistent infections, and intrafamily strain variation (39).

High mean genetic diversity. The identification of a high level of allelic variation among isolates classified as *H. pylori* is consistent with data generated by several other genetic techniques. However, our studies extend earlier analyses by the discovery that the level of metabolic enzyme allelic variation exceeds that of other human, animal, and plant bacterial pathogens (Table 2). The substantial genetic diversity in natural populations could be due to several factors such as large population size, high mutation frequency, or unusually high levels of recombinational activity.

In recent years there has been increasing interest in the application of large-scale DNA sequencing strategies to studies of molecular variation in microbial populations. Thus far, the entire genomes of one isolate each of two bacterial pathogens (*Haemophilus influenzae* and *Mycoplasma genitalium*) have been sequenced (17, 21). Inasmuch as this study demonstrates that isolates classified as *H. pylori* are highly polymorphic in allelic variation at metabolic-enzyme-encoding loci, it is likely that considerable nucleotide sequence diversity will exist at virtually any locus studied. Consistent with this idea is the observation that there is substantial variation in the genome size of *H. pylori* strains recovered from patients (57) and in DNA-DNA reassociation values (64). Moreover, recent data demonstrate that the physical maps of five *H. pylori* isolates are very different, as assessed by variance in the locations of 17 structural genes (27). These observations mean that when a complete genomic sequence becomes available for an *H. pylori* isolate, it must be interpreted with the knowledge that there is substantial genetic variation in the species. Stated another way, the sequence should be thought of as an *H. pylori* genome sequence, rather than the genome sequence.

Because DNA sequence data are not available for the genes encoding the allozymes identified in this analysis, the nature and extent of amino acid variation responsible for the observed enzyme electromorphs are unknown. However, Boyd et al. (6) demonstrated that for malate dehydrogenase in *Escherichia coli* and *Salmonella enterica*, on average, the generation of a new allozyme has involved 2.6 amino acid substitutions. This suggests that considerable amino acid sequence variation exists among allelic variants of the metabolic enzymes we studied and implies that proteins encoded by other loci will also be characterized by high levels of polymorphism.

High frequency of occurrence of null alleles. In addition to the large mean number of alleles per locus, our study also identified an unexpected unusually high frequency of occurrence of null alleles. Several explanations are possible for the lack of detectable enzyme activity, including the absence of all or part of the structural gene, downregulation of enzyme production, occurrence of enzyme-inactivating mutations, etc. Currently, we lack sufficient data to differentiate among the many hypotheses. However, our data are noteworthy in the context of the observation by Phadnis et al. (45) and Covacci et al. (13) and Telford et al. (60), that some strains lack the vacuolating toxin gene and cytotoxin-associated gene A, respectively. Taken together, the data suggest that the absence of

TABLE 3. Mean genetic diversity per locus in *H. pylori* recovered from different clinical categories

| Disease | No. of isolates | No. of ETs | Mean no. of alleles | Mean diversity (<i>H</i>) |
|----------------|-----------------|------------|---------------------|-----------------------------|
| Gastritis | 30 | 29 | 7.3 | 0.698 |
| Gastric ulcer | 7 | 7 | 4.7 | 0.810 |
| Duodenal ulcer | 37 | 36 | 9.3 | 0.763 |

enzyme activity in some strains may be due to deletion of all or part of the structural gene.

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