A Comparison of 16S Ribosomal DNA Sequences from Five Isolates of *Helicobacter pylori*

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Other workers have found that clinical isolates of *Helicobacter pylori* exhibit very extensive DNA sequence polymorphisms when they are examined by ribotyping or some other genomic sequence characterization technique. In fact, it is rare to find similar clones, much less identical ones, among isolates. We found that the levels of divergence between the 16S ribosomal DNA sequences of individual organisms and the consensus sequence of the five isolates which we examined ranged from 0.2 to 0.5%. In contrast, other workers have shown that levels of divergence between the 16S ribosomal DNA sequence of *H. pylori* and the 16S ribosomal DNA sequences of four other *Helicobacter* species range from 2.7 to 8.0%. Our results show that the *H. pylori* 16S ribosomal DNA is not very polymorphic and support the conclusion that *H. pylori* is a unique species.

Strains of *Helicobacter pylori*, the suspected agent of type B gastritis and gastric and duodenal ulcers, appear to exhibit high levels of genetic variability. The possibility that such variability occurs was suggested by the results of clonal analyses of 134 isolates by molecular techniques (1, 3, 7, 8, 10). In the study of Taylor et al. (10), genomic DNAs from 20 isolates were examined for restriction fragment length polymorphism patterns and only two patterns matched. Perhaps more remarkably, the sizes of only a minority of the fragments matched. Similarly, Linton et al. (7) used ribotyping to show that none of the 12 strains that they examined were identical. These authors observed from 50 to 70% dissimilarity in fragment sizes. The results of these two studies are consistent with the results of Akopyanz et al. (1), who used random amplification of a single small primer to map the sequence sites in *H. pylori* genomes. In this study, 64 isolates were examined, and no 2 isolates gave the same pattern of amplicons. Again, the patterns of amplicon sizes were highly variable.

Beyond academic interests, several important, medically related questions are raised by these findings. One question concerns the utility of sequence information for identification. A second question is whether organisms clinically classified as *H. pylori* are correctly identified since the number of phenotypic parameters used in routine diagnostic examinations is limited. For example, any microaerophilic, gram-negative, curved organism that produces urease, oxidase, and catalase and is isolated from a human gut is classified as *H. pylori*. Might there be several species of human pathogens that fit this profile? We know that there are several *Helicobacter* species whose phenotypes fit this profile but are distinguished as separate species by other factors, and one of the major characteristics is 16S ribosomal DNA sequence data (4).

Bacterial phylogeny and identification are coming to rest squarely on ribosomal sequence information. Thus, the third important question is whether the extensive polymorphism observed in genomic sequences and ribotypes of *H. pylori* extends to 16S ribosomal DNA sequences and, if so, to what degree. To begin to answer to these questions, we amplified

and sequenced the 16S ribosomal genes of five strains of *H. pylori*.

MATERIALS AND METHODS

Bacteria. Five isolates of *H. pylori* were examined. One isolate was strain ATCC 43504, and the other four isolates were clinical isolates recovered from endoscopic biopsies of patients evaluated at the Mayo Clinic in Rochester, Minn. The latter four isolates were designated MC123, MC268, MC903, and MC937. The nonidentity of the isolates was verified in our laboratories by Susan Wilkinson, who used pulsed-field gel electrophoresis performed as described by Taylor et al. (10) and genomic DNAs digested with *Nru*I (data not shown).

DNA sequencing. Our sequencing strategy was to make 16S amplicons from purified *H. pylori* DNA by using universal primers p246 and pH (Table 1) and the PCR. DNA was purified from approximately 5×10^8 cells in the exponential phase of growth by using phenol-chloroform extraction (9). Each 50- μ l PCR mixture contained 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 200 μ M dATP, 200 μ M dCTP, 200 μ M dGTP, 200 μ M dTTP, 50 pmol of each primer, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus), and 1 μ g of extracted *H. pylori* DNA. The cycling conditions used were denaturation for 1 min at 94°C, annealing for 2 min at 50°C, and extension for 3 min at 72°C for 35 cycles, followed by a final extension for 10 min at 72°C. An identical mixture lacking *H. pylori* target DNA was used as a negative control in each amplification experiment. The 16S amplicons were purified by using PCR Magic Prep columns (Promega) according to the manufacturer's instructions. A 5- μ l portion of each purified amplicon was sequenced by using a dye terminator reagent kit, including *Taq* polymerase, and the protocol recommended by the manufacturer (Applied Biosystems, Inc., Foster City, Calif.); 3.2 pmol of sequencing primer was used per reaction mixture, and the cycling conditions used were denaturation at 96°C for 30 s, annealing at 50°C for 15 s, and extension for 4 min at 60°C for 25 cycles. The sequencing reaction products were separated from dye-labelled precursors by using Centri-Sep columns (Princeton Separations, Inc., Adelphia, N.J.) according to the manufacturer's instructions. The sequencing reaction products were then analyzed with a model 373A automated DNA sequencer (Applied Biosystems, Inc.).

Nucleotide sequence accession numbers. The nucleotide

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TABLE 1. *H. pylori* 16S ribosomal DNA sequencing primers^a

Primer	Sequence	Positions
Forward primers		
p246	5' AGA GTT TGA TCC TGG CTC AG 3'	8-27
p170	5' TAA TAC CAG ATA CTC .CT ACG G 3'	170-191
p392	5' GGA GGA TGA AGG TTT TAG GAT 3'	392-412
p548	5' GGG ATA GTC AGT CAG GTG TG 3'	548-567
p693	5' CGA CCT GCT GGA ACA TT 3'	693-709
p903	5' CGG TGG AGC ATG TGG TTT AAT 3'	903-923
p1136	5' TAA GGA GGA GGA AGG TG 3'	1136-1152
p1342	5' AAT ACG TTC CCG GGT CTT GT 3'	1342-1361
Reverse primers		
p71R	5' CAT CGT TCG ACT TGC ATG TA 3'	71-52
p192R	5' CCC GTA .GG AGT ATC TGG TAT 3'	192-172
p275R	5' ATA CCC GTC ATA GCC TTG GT 3'	275-256
p412R	5' ATC CTA AAA CCT TCA TCC TC 3'	412-393
p663R	5' TAC GGA TTT TAC CCC TAC AC 3'	663-644
p1073R	5' TCG TTG CGG GAC TTA ACC CAA 3'	1073-1053
p1392R	5' ATG GTG TGA CGG GCG GTG AG 3'	1382-1363
pH	5' AAG GAG GTG ATC CAG CCG CA 3'	1484-1503

^a Primers p246 and pH are universal 16S ribosomal DNA primers taken from the study of Edwards et al. (5). The periods in primers p170 and p192R represent inadvertent deletion of a C in the oligonucleotides compared with the sequence shown in Fig. 1. Despite the deletion, the primers worked effectively. The positions of the primers refer to the sequence numbers used in Fig. 1.

sequences of the isolates which we used have been deposited in the GenBank data base under the following accession numbers: strain MC123, U01328; strain MC238, U01329; strain ATCC 43504, U01330; strain MC937, U01331; and strain MC903, U01332. The *H. pylori* ATCC 43504 sequence determined by Eaton et al. (4) was deposited in the GenBank data base under accession number M88157, and the sequences of the seven strains studied by Höök-Nikanne et al. (6) were deposited in the GenBank data base under accession numbers M55303 to M55309.

RESULTS AND DISCUSSION

H. pylori has been reported to contain three copies of the 16S ribosomal gene (10); we did not determine the numbers of copies in the isolates which we studied. To sequence the 16S ribosomal genes, we used universal PCR primers and genomic DNA to produce amplicons. The amplicons were then partially purified and sequenced by using dye-labelled dideoxy terminators, the Sanger technique, and an automated sequencer. Within the sensitivity of this method, all of the copies that were amplified appeared to be identical because we did not detect peaks that were obvious mixtures of bases. A possible exception was at position 1434, as discussed below.

Figure 1 shows the consensus sequence determined for the five isolates. The sequence of each individual isolate can be deduced by altering the consensus sequence at the positions indicated in Table 2. As Table 2 shows, deviations from the consensus sequence were found at 13 separate positions. The number of deviations from the consensus sequence were two (strains ATCC 43504 and MC930), three (strains MC123 and MC937), and five (strain MC268). We found that strains MC123 and MC43504 share a common variation, a C → T transition at position 1105, while strains MC903 and MC937 also share a common variation, an A → G transition at position 993. Since each of these strains has only two or three differences, the sharing of a common change suggests a clonal origin. However, more information concerning these isolates is needed before firm conclusions can be drawn. Table 2 also shows that the majority of variation occurs from about position 801 to position 1419; that is, 69% of the variation occurs in the 3' one-half of the gene. The variation that occurs in the 5' end of the gene is clustered between positions 91 and 95. Of the 13 variants, 11 represent transitions; the change at 95 is a T → G transversion, and the change at position 92 is a single-base deletion.

The 16S ribosomal DNA sequence of *H. pylori* ATCC 43504 is 97.4% identical to the sequence of *Helicobacter acinonyx*;

TABLE 2. 16S ribosomal DNA sequence differences in five *H. pylori* strains

Strain	Base at position:													No. of differences compared with consensus sequence	No. of differences compared with sequence of strain:			
	91	92	94	95	801	993	1000	1105	1235	1245	1259	1411	1419		MC123	ATCC 43504	MC903	MC937
MC123								T				A		2				
ATCC 43504	G							T	T					3	3			
MC903						G				T				2	4	5		
MC937					A	G	C							3	5	6	3	
MC268		Δ ^a	T	G							G		A	5	7	8	7	8
Consensus	A	T	C	T	G	A	T	C	C	C	A	G	G					

^a Δ, deletion.

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1  TTTATGGAGA GTTGTATCCT GGCTCAGAGT GAACGCTGGC GGCCTGCCTA
51  ATACATGCAA GTCGAACGAT GAAGCTTCTA GCTTGTCTAG ATGCTGATTA
101 GTGGCGCAGC GGTGAGTAAC GCATAGGTCA TGTGCCTCTT AGTTTGGGAT
151 AGCCATTGGA AACGATGATT AATACCAGAT ACTCCCTACG GGGGAAAGAT
201 TTATCGCTAA GAGATCAGCC TATGTCTCTAT CAGCTTGTGT GTAAGGTAAT
251 GGCTTACCAA GGCTATGACG GGTATCCGGC CTGAGAGGGT GAACGGACAC
301 ACTGGAACCT AGACACGGTC CAGACTCCTA CGGGAGGCAG CAGTAGGGAA
351 TATTGCTCAA TGGGGGAAAC CCTGAAGCAG CAACGCCGCG TGGAGGATGA
401 AGGTTTTAGG ATTGTAACT CTTTTGTGA GAGAAGATAA TGACGGTATC
451 TAACGAATAA GCACCGGCTA ACTCCGTGCC AGCAGCCGCG GTAATACGGA
501 GGGTGCAAGC GTTACTCGGA ATCACTGGGC GTAAGAGCG CGTAGGCGGG
551 ATAGTCAGTC AGGTGTGAAA TCCTATGGCT TAACCATAGA ACTGCATTTG
601 AAATACTAT TCTAGAGTGT GGGAGAGGTA GGTGGAATTC TTGTGTAGG
651 GGTAAATACC GTAGAGATCA AGAGGAATAC TCATTGCGAA GGCACCTGCT
701 TGGAACATTA CTGACGCTGA TTGCGCGAAA GCGTGGGGAG CAAACAGGAT
751 TAGATACCCT GGTAGTCCAC GCCCTAAACG ATGGATGCTA GTTGTGGAG
801 GGCTTAGTCT CTCAGTAAT GCAGTAACG CATTAAGCAT CCCGCCTGGG
851 GAGTACGGTC GCAAGATTAA AACTCAAAGG AATAGACGGG GACCCGCACA
901 AGCGGTGGAG CATGTGGTTT AATTGGAAGA TACACGAAGA ACCTTACCTA
951 GGCTTGACAT TGAGAGAATC CGTAGAATAT AGTGGAGTGT CTAGCTTGCT
1001 AGACCTTGAA AACAGGTGCT GCACGGCTGT CGTCAGCTCG TGTCGTGAGA
1051 TGTGGGTTA AGTCCGCAA CGAGCGCAAC CCCCTTTCTT AGTTGCTAAC
1101 AGGTCATGCT GAGAACTCTA AGGATACTGC CTCGTAAGG AGGAGGAAGG
1151 TGGGGACGAC GTCAAGTCAT CATGGCCCTT ACGCCTAGGG CTACACACGT
1201 GCTACAATGG GGTGCACAAA GAGAAGCAAT ACTGCGAAGT GGAGCCAATC
1251 TTCAAACAC CTCTCAGTTC GGATTGTAGG CTGCAACTCG CCTGCATGAA
1301 GCTGGAATCG CTAGTAATCG CAAATCAGCC ATGTTGCGGT GAATACGTTT
1351 CCGGGTCTTG TACTCACCGC CCGTCACACC ATGGGAGTTG TGTTTGCTTT
1401 AAGTCAGGAT GCTAAATTGG TCTACTGCCA CGGCACACAC AGCGACTGGG
1451 GTGAAGTCGT AACAAGGTAA CCGTAGGTGA ACCTGCGGCT GGATCACCTC
1501 CTT

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FIG. 1. Consensus sequence of *H. pylori* 16S ribosomal DNA. This sequence represents a compilation of the data for the five isolates discussed in the text and shown in Table 2 plus the data for the GenBank accession number M88157 sequence deposited by Eaton et al. (4). The strain ATCC 43504 sequence and the GenBank accession number M88157 sequence are the same, as described in the text. The sequences of all of the isolates are identical to the consensus sequence except as indicated in Table 2. The numbers begin as described by Brosius et al. (2) for the *Escherichia coli* 16S ribosomal gene. We determined the sequences for all isolates on both strands in their entirety except for the region from position 1327 to position 1378; for this region we could determine the sequence for only one strand. However, our sequence agrees perfectly with the sequence of Eaton et al. (4). The forward and reverse PCR primers (primers p246 and pH) are underlined. The portion of the sequence in boldface type is new data.

that is, the two sequences differ at almost 30 positions (4). It is interesting that because of mutations at positions 91, 94, and 95 strain MC268 has the same sequence as *H. acinonyx* (that is, AGAAGTGGA). Also, the mutation at position 1419 in strain MC268 changes a G to an A. A is also found at position 1419

in *H. acinonyx*. Thus, four of the five differences between the strain MC268 sequence and the consensus sequence are found in *H. acinonyx*. However, *H. acinonyx* still differs from strain MC268 at approximately 25 other positions.

Our results are largely in agreement with those of Eaton et al., whose unpublished *H. pylori* ATCC 43504 sequence has been deposited in the GenBank data base. These authors used rRNA and reverse transcriptase to make single-stranded DNA for sequencing. Our sequence differs from the sequence of Eaton et al. at two locations; C is missing from the sequence of Eaton et al. at our position 184, and we found a G at our position 727, whereas Eaton et al. found a T. The results of a reexamination by Eaton et al. of their raw data suggest that our results are correct (3a). In various experiments we observed an A or C at our position 1434, but Dewhirst and colleagues (3b) have shown that there is always a C at this position in *Helicobacter* species.

Previously, Höök-Nikanne et al. (6) sequenced 397 bp of 16S RNAs of seven *H. pylori* strains isolated on three continents. The region which these authors sequenced corresponds to our coordinates 71 to 468 (Fig. 1). We examined the data of Höök-Nikanne et al. (which have been deposited in the GenBank data base) and compared their results with to our results. The data agreed completely with the following exceptions: (i) like Eaton et al., Höök-Nikanne et al. did not find C at position 184; (ii) at position 71 we found only G, and Höök-Nikanne et al. found only C; and (iii) Höök-Nikanne et al. reported three single-base-pair differences from the consensus sequence in two strains, at positions 91 (T → G), 129 (C → T), and 463 (A → T). We also found a change at position 91 in one strain; however, the change which we found was a deletion (Table 2).

In addition to the differences between our results and those of other workers, we were able to identify the unknown bases in GenBank sequence M88157 at our positions 913, 928 to 931, and 1084 (Fig. 1). Moreover, we also sequenced an additional 30 bp at the 3' end of the 16S DNA sequence (Fig. 1, boldface type) beyond the bases reported in GenBank sequence M88157 even though we examined an additional 50 bp; we did not count the last 20 bp because this region represented primer H, which was used to make the amplicon.

The results of a large number of studies have established the utility and validity of microbial phylogeny based on 16S DNA sequence data (see reference 11 for a review). For the most part, 16S DNA sequence data represent sequence information obtained from a single isolate of a species. Indeed, this type of data base should be useful for clinical laboratories as well. However, the following immediate questions occur: to what extent do clinical specimens deviate from the data base entry, and how much deviation is acceptable? Our results address the first question but not the second.

Deviation can be assessed by comparison with a type strain, by pairwise comparisons (Table 2), or by comparison with a consensus sequence (Table 2). While each of these approaches has its merits, comparison with a consensus sequence minimizes disparity and avoids distortion due to selection of an arbitrary standard. Presumably, the consensus sequence is closer to the *H. pylori* ancestral sequence than the sequence of any given isolate. Since the sample size in this study was small (five isolates), we doubt that our *H. pylori* consensus sequence is the final one. In fact, partial sequencing results for an additional five isolates indicated that A and G occur equally at our position 91. Thus, the consensus sequence seems to have a purine at this position. Our results clearly establish that 16S ribosomal DNA sequences in *H. pylori* are relatively stable and that the high level of genomic variability ascribed to this

organism does not extend to the 16S ribosomal genes. Indeed, the sequences of the five *H. pylori* strains differ from the consensus sequence by no less than 0.2% and no more than 0.5%; in contrast, five *Helicobacter* species, including *H. pylori*, *H. acinonyx*, *Helicobacter felis*, *Helicobacter mustalae*, and *Helicobacter muridarum*, differ in their 16S ribosomal DNA sequences by 2.7 to 8.0% (4). Thus, our data support the notion *H. pylori* is a separate species. Finally, we raised the possibility above that the morphological and physiological parameters used clinically to isolate and identify *H. pylori* might not be as discerning as molecular data. Thus far, our results do not support this possibility.

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