Phylogenetic analysis of gastric and enterohepatic *Helicobacter* species based on partial HSP60 gene sequences

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Analysis of 16S rRNA gene sequences has been the method generally used to study the evolution and phylogeny of bacteria. Phylogenetic analysis of the 16S rRNA gene has shown the position of the genus *Helicobacter* in the ϵ -subclass of the *Proteobacteria*. Because 16S rRNA-based phylogeny does not always correspond to the results of polyphasic taxonomy, and the related species cannot always be separated, new phylogenetic markers for *Helicobacter* species are needed. In this study, conserved partial (600 bp) 60 kDa heat-shock protein (HSP60) sequences were used to study the phylogeny of 37 strains of gastric and enterohepatic *Helicobacter* species, including type strains of 15 *Helicobacter* species with validly published names, reference strains of flexispira taxa and *Helicobacter felis*, *Helicobacter bizzozeronii* and *Helicobacter salomonis* and canine flexispira strains. The partial HSP60 gene sequence proved to be a useful phylogenetic marker for the genus *Helicobacter*, providing a means of differentiating all 15 *Helicobacter* species analysed. In the resulting phylogenetic tree, gastric *Helicobacter* species and enterohepatic species with flexispira morphology formed tight, separate clusters. In general, HSP60 sequence similarities between *Helicobacter* species were significantly lower than the corresponding 16S rRNA gene sequence similarities, indicating a better resolution for species identification. In addition, a specific PCR method for identifying *H. salomonis* was developed based on the partial HSP60 sequence.

INTRODUCTION

Helicobacter species have been isolated from the gastrointestinal tract of humans and several animal species, including cats, dogs, monkeys, sheep, pigs, rodents, birds, cheetahs and poultry (On, 1996, 2001; Solnick & Schauer, 2001). At the time of writing (September 2003; <http://www.bacterio.cict.fr>), there were 22 *Helicobacter* species with validly published names and two uncultured 'Candidatus' species (Dewhirst *et al.*, 2000b). Several other *Helicobacter* taxa remain unnamed, because they have not been properly described according to the internationally accepted rules of nomenclature. Flexispiras constitute one of the unnamed groups of *Helicobacter* species, sharing spindle-shaped cell morphology and periplasmic fibrils (Bryner *et al.*, 1987). The group includes three species with validly published names, *Helicobacter bilis* (Fox *et al.*, 1995),

Helicobacter trogontum (Mendes *et al.*, 1996; Hänninen *et al.*, 2003) and *Helicobacter aurati* (Patterson *et al.*, 2000), plus several unnamed taxa (on the basis of analysis of 16S rRNA gene sequences) (Dewhirst *et al.*, 2000a).

Classification of helicobacters has been hampered by difficulties in growing them *in vitro* and by their inertness in traditionally used biochemical tests. Culture-independent genetic methods, such as 16S rRNA gene sequence analysis, have therefore been applied to study the phylogeny of the genus as a basis for classification (Dewhirst *et al.*, 2000a; Jalava *et al.*, 1997; On, 2001). The results from 16S rRNA gene analyses are not always concordant with those of polyphasic taxonomy. For example, *Helicobacter* species flexispira 16S rRNA taxon 1, 4, 5 and 6 reference strains were widely scattered in the 16S rRNA-based phylogenetic tree (Dewhirst *et al.*, 2000a), but, according to the polyphasic taxonomy, they were all highly similar and shown to be members of *H. trogontum* (Hänninen *et al.*, 2003). Similarly, *Helicobacter cinaedi* has a high degree of divergence in its 16S rRNA gene sequences (Vandamme *et al.*, 2000). For most *Helicobacter* species, the intraspecific divergence of the 16S rRNA gene is not known.

Phylogenetic analysis of *Helicobacter* species based on urease

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Abbreviation: HSP60, 60 kDa heat-shock protein.

The GenBank/EMBL/DDBJ accession numbers for the *Helicobacter* HSP60 gene sequences determined in this study are AJ558199–AJ558235.

α -subunit amino acid sequences gave a result similar to that for 16S rRNA gene analysis, but phylogenetic analysis is restricted to urease-positive *Helicobacter* species (Gueneau & Loiseaux-De Goër, 2002). The HSP60 (60 kDa heat-shock protein) gene (GroEL, chaperonin), which encodes a 60 kDa subunit of a complex assisting the three-dimensional folding of bacterial proteins (Fink, 1999), has the potential to serve as a general phylogenetic marker because of its ubiquity and conservation in nature (Segal & Ron, 1996). Studies on the suitability of a fragment from a conserved region of the HSP60 gene for phylogenetic analyses and speciation of the genera *Staphylococcus* and *Macroccoccus* (Goh *et al.*, 1996; Kwok & Chow, 2003) and *Bacteroides* (Jian *et al.*, 2001) have been published. These studies have shown that, despite the conserved nature of the HSP60 gene, interspecies variation in the DNA sequences is greater than that in the corresponding 16S rRNA gene sequences, which may therefore provide better resolution for species classification.

The molecular methods used recently for identification of novel *Helicobacter* isolates include 16S and 23S rRNA PCR-RFLP and species-specific PCR methods (Shen *et al.*, 2000; Hurtado & Owen, 1997). However, differentiation of the three closely related *Helicobacter* species *Helicobacter felis*, *Helicobacter bizzozeronii* and *Helicobacter salomonis* is not possible by these methods and requires a combination of whole-genome DNA–DNA hybridization and phenotypic analysis (Jalava *et al.*, 1997).

In this study, our aim was to investigate the phylogeny of the genus *Helicobacter*, including some unnamed flexispira isolates, using 600 bp partial HSP60 gene sequences. Our other goal was to develop a PCR method based on this gene fragment to differentiate the three closely related species *H. felis*, *H. bizzozeronii* and *H. salomonis*.

METHODS

Bacterial strains. Thirty-seven strains, including type and reference strains of various *Helicobacter* species and flexispira taxa, *H. felis*, *H. bizzozeronii* and *H. salomonis* strains and some Finnish canine flexispira strains, were used to study the phylogeny of partial HSP60 sequences (Table 1).

DNA isolation and PCR amplification. Bacteria were cultured on Brucella blood agar plates (Oxoid) containing selective antimicrobial agents (Hänninen *et al.*, 1996) and grown microaerobically at 37 °C for 2–3 days. Bacterial mass was collected from two or three plates and genomic DNA was isolated by the method of Pitcher *et al.* (1989) as described previously (Hänninen *et al.*, 1996). The partial HSP60 gene sequence was amplified with the degenerate primers H60F (5'-GGNGAYGGNCANCANCANGCNCANGT-3') and H60R (5'-TCNCCRAANCCNGGNGCYTTNCANGC-3'). The amplified region corresponds to nt 1345–1935 of the GroEL-like protein in *Clostridium perfringens* (Rusanganwa *et al.*, 1992). The 50 µl PCR consisted of 1–3 µl purified DNA, 5 nmol each dNTP, 2.5 U *Taq* DNA polymerase (MBI Fermentas), 5 µl buffer without MgCl₂, 75 nmol MgCl₂ and 100 pmol each primer. The PCR thermal cycling conditions were as described previously (Jian *et al.*, 2001).

Cloning and sequencing of PCR products. PCR products of the expected size were purified from 2% NuSieve GTG low-melting agarose gel (BioWhittaker Molecular Applications) with the Qiaquick gel extraction kit (Qiagen), cloned into pGEM-T Easy vector (Promega) and transformed to competent *Escherichia coli* JM109 cells (Promega). Ampicillin (150 µg ml⁻¹) and blue/white selection were used to choose the transformants. Plasmids were isolated from several clones of each transformation with the Qiagen plasmid-purification Mini kit. To confirm the presence of the correct insert, plasmids were digested with *Eco*RI and restriction products were then separated in 1% agarose gel. Nucleotide sequencing of three cloned fragments was performed by automated cycle sequencing with Big Dye terminators (ABI 377XL; PE Applied Biosystems).

Sequence analysis. The partial HSP60 sequences determined in this study and those available in public databases [*Helicobacter pylori* CH-CTX1 (accession no. AF479030), *H. pylori* CCUG 17874 (X73840), *H. pylori* J99 (AE001439), *H. pylori* 26695 (AE000511), *Campylobacter jejuni* NCTC 11168 (AY044099) and *Campylobacter coli* NCTC 11353 (AY044098)] were analysed with BIONUMERICS software (version 3.0; Applied Maths). After pairwise and multiple alignments, a phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987) and the Jukes–Cantor coefficient (Jukes & Cantor, 1969). The topology of the tree was evaluated by performing 1000 trials of bootstrap analysis. The DNA sequences were translated to the corresponding protein sequences with the TRANSEQ program (EMBOSS; European Molecular Biology Open Software Suite). The resulting protein sequences were aligned and a neighbour-joining tree calculated with CLUSTAL W (Thompson *et al.*, 1994). The phylogenetic tree based on the protein sequences was drawn using TREEVIEW version 1.6.6 (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

Species-specific PCR for differentiating *H. felis*, *H. bizzozeronii* and *H. salomonis*. Specific primers for amplifying a partial HSP60 sequence from *H. felis*, *H. bizzozeronii* or *H. salomonis* were designed with the PRIMER3 program and tested against the strains of three species by using the PRIMERSEARCH program (EMBOSS). The primer pairs were tested in the laboratory with five representative strains from each of the three species. The PCR cycling program for the specific primers was the same as that for the degenerate H60F and H60R primers except that, after optimization experiments, the annealing temperature was raised to 66 °C.

RESULTS AND DISCUSSION

Phylogenetic analysis of DNA sequences

A phylogenetic tree based on the partial HSP60 sequences was constructed (Fig. 1). The topology of the neighbour-joining tree was basically similar to those of trees based on 16S rRNA or urease α -subunit amino acid sequences (Dewhirst *et al.*, 2000a; Gueneau & Loiseaux-De Goër, 2002), with gastric species forming a tight cluster (cluster 1) and enterohepatic species a more diverse one (cluster 2). Interspecies sequence similarity values ranged from 61.4 to 94.2% (mean 72%), showing much higher variation than that in 16S rRNA gene sequences, where the similarities between *Helicobacter* species ranged from 88.7 to 99.2% (Jalava *et al.*, 1997; Dewhirst *et al.*, 2000a). In the genus *Bacteroides*, interspecies similarity of the respective sequences ranged from 73 to 96% (mean 85%) (Jian *et al.*, 2001). These results support the idea that the HSP60 gene

Table 1. *Helicobacter* strains used in this study

Taxon	Strain/reference	Source
<i>H. acinonychis</i>	90-1908 (=ATCC 51104)	Cheetah gastric mucosa
<i>H. bizzozeronii</i>	Storkis ^T (=CCUG 35545 ^T)	Canine gastric mucosa
	Emo (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa
	10 (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa
	Yrjälä (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa
	R-53 (Jalava <i>et al.</i> , 2001)	Human gastric mucosa
<i>H. canis</i>	NCTC 12739 ^T	Canine faeces
<i>H. cinaedi</i>	CCUG 18818 ^T	Human faeces
<i>H. cholecystus</i>	CCUG 38167	Syrian hamster gall-bladder
<i>H. felis</i>	CS1 (=ATCC 49179)	Feline gastric mucosa
	DS3 (Paster <i>et al.</i> , 1991)	Canine gastric mucosa
	Kukka (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa
	Lopki13 (Jalava <i>et al.</i> , 1998)	Feline gastric mucosa
<i>H. hepaticus</i>	CCUG 33637 ^T	Mouse liver
<i>H. muridarum</i>	CCUG 29262 ^T	Rat ileum
<i>H. mustelae</i>	CCUG 25715 ^T	Ferret gastric mucosa
<i>H. pametensis</i>	CCUG 29253	Tern faeces
<i>H. pullorum</i>	NCTC 12826	Human faeces
<i>H. pylori</i>	Tx30a (=ATCC 51932)	Human gastric mucosa
	7546	Human gastric mucosa
<i>H. salomonis</i>	Inkinen ^T (=CCUG 37845 ^T)	Canine gastric mucosa
	06A (=CCUG 37848)	Canine gastric mucosa
	HS100	Canine gastric mucosa
	Ko. K. III (Jalava <i>et al.</i> , 1998)	Canine gastric mucosa
<i>H. trogonum</i>	HU H95S (Hänninen <i>et al.</i> , 2003)	Pig faeces
<i>H. trogonum</i>	86-13625 (=ATCC 43968)	Pig
<i>Helicobacter</i> sp., taxon 2	86-2279 (=ATCC 49314)	Sheep
<i>Helicobacter</i> sp., taxon 3	88-2491 (=ATCC 49320)	Pig stomach; aborted fetus
<i>H. trogonum</i>	86-1775 (=ATCC 49310)	Sheep
<i>H. trogonum</i>	84-3345 (=ATCC 43966)	Aborted sheep fetus
<i>H. trogonum</i>	ATCC 700114 ^T	Rat gut
<i>Helicobacter</i> sp., taxon 8	CCUG 23435	Human faeces
<i>H. bilis</i>	ATCC 51630 ^T	Mouse faeces
Canine 'flexispira'	FL56	Canine gastric mucosa
	KO214	Canine gastric mucosa
	KO220	Canine gastric mucosa
	KO534B	Canine gastric mucosa

would give better resolution for species identification than the 16S rRNA gene. The high similarity of the overall structures of the phylogenetic trees based on 16S rRNA, urease α -subunit and partial HSP60 sequences reveals the early division of the species into gastric and enterohepatic lineages. The enterohepatic species *Helicobacter canis*, *Helicobacter cholecystus*, *Helicobacter pametensis*, *H. cinaedi*, *Helicobacter hepaticus*, *Helicobacter pullorum* and *Helicobacter mustelae* remained outside these two clusters. These species formed no distinct or stable cluster(s) in the tree (Fig. 1).

The topology of the gastric cluster (cluster 1) was also similar to the one in the 16S rRNA analysis (Dewhirst *et al.*, 2000a; Gueneau & Loiseaux-De Goër, 2002) and it was further subdivided, with high bootstrap support, into two

subclusters (subclusters 1 and 2). Sequence similarities between species in cluster 1 ranged from 75.8 to 94.3% (mean 83.2%). Subcluster 1 included the six *H. pylori* strains and *Helicobacter acinonychis*, and these two species were clearly separated from each other (with a sequence similarity in the range 93.8–95.0%). HSP60 DNA sequence similarities between the six *H. pylori* strains ranged from 96.6 to 98.3% (mean 97.3%), demonstrating that the intraspecies similarity within subcluster 1 is higher than the interspecies similarity. Both comparison of several 16S rRNA gene sequences (Eckloff *et al.*, 1994) and the use of other sequence-based genotyping methods (Maggi Solcà *et al.*, 2001) have revealed high diversity for *H. pylori*. Subcluster 2 included gastric species *H. salomonis*, *H. bizzozeronii* and *H. felis*. *H. salomonis* strains were separated from *H. felis* and *H. bizzozeronii* with strong bootstrap

Fig. 1. Phylogenetic tree based on partial HSP60 DNA sequences. The tree was constructed by use of the neighbour-joining method and rooted with sequences from *Campylobacter coli* and *Campylobacter jejuni*. Numbers at branch-points represent percentage bootstrap support calculated from 1000 trees. The scale bar represents the sequence divergence of sequences.

support. Interspecies similarity within subcluster 2 was in the range 88.1–93.2 %, revealing higher diversity than that within subcluster 1. Interstrain similarity was high in *H. salomonis* (mean 99.7 %). *H. felis* and *H. bizzozeronii* had more divergent sequences, respectively showing approximately 95.1 % (range 93.8–97.4 %) and 95.4 % (range 93.2–99.6 %) interstrain similarity. The 16S rRNA gene sequences of two *H. salomonis* strains differed by 1.7 % (Jalava *et al.*, 1997), indicating higher variability than for HSP60 sequences. The human *H. bizzozeronii* strain R-53, previously known as '*Helicobacter heilmannii*' type 1, clearly clustered with canine *H. bizzozeronii* strains, thus confirming the results based on the polyphasic taxonomic studies of the strain (Jalava *et al.*, 2001).

Cluster 2 included all enterohepatic strains with flexispira morphology: *H. bilis* and *Helicobacter muridarum* type strains, five *H. trogonum* strains, reference strains of *Helicobacter* sp. flexispira taxa 2, 3 and 8 and four Finnish canine flexispira isolates (KO534B, KO220, KO214 and F56). *H. trogonum* strains grouped tightly together and were separate from the other species. The sequence similarity between the two species *H. bilis* and *H. trogonum* ranged from 88.4 to 88.9 % (mean 88.6 %). Three of the canine isolates (KO534B, KO220 and KO214) clustered tightly (mean 98.4 % similarity) with *H. bilis* and the taxon 8 reference strain. *H. bilis* has been isolated from several animal species, including mouse, dog, hamster, gerbil and

rat (Fox *et al.*, 1995; Solnick & Schauer, 2001). Our present results, as well as the results of cytolethal distending toxin gene sequencing (Kostia *et al.*, 2003), suggest a close relationship between *H. bilis* and canine flexispiras, but further studies are needed to confirm the taxonomy of the latter. Reference strains of taxa 2 and 3 were located in their own branch, as was the canine isolate FL56.

In the 16S rRNA analysis, *H. trogonum* strains were widely dispersed among enterohepatic *Helicobacter* species (Dewhirst *et al.*, 2000a; Hänninen *et al.*, 2003). For example, strains ATCC 43968 and ATCC 49310 differed by 4.4 % in their 16S rRNA gene sequences (Hänninen *et al.*, 2003) but showed only 0.6 % difference in the partial HSP60 sequences. Between strains ATCC 43968 and ATCC 43966, the corresponding differences were respectively 4.7 % (Dewhirst *et al.*, 2000a; Hänninen *et al.*, 2003) and 0.6 %. These results show that, in the case of *H. trogonum*, HSP60 gene phylogeny predicts taxonomy much better than does the 16S rRNA gene. In general, our studies revealed that the evolution of the 16S rRNA and HSP60 genes is not similar, 16S rRNA being more often a target of genetic changes.

Although the enterohepatic species *H. pullorum*, *H. cholecystus*, *H. pametensis*, *H. cinaedi*, *H. hepaticus* and *H. canis* did not cluster together, they were more similar to each other (mean interspecies similarity 75.4 %) and other

enterohepatic species in cluster 2 than to any species in cluster 1 (mean interspecies similarity 69.3%). All these species, except *H. hepaticus*, are urease-negative. This characteristic could suggest the intestine as the primary niche for colonization, because all gastric *Helicobacter* species are urease-positive (Solnick & Schauer, 2001). The HSP60 sequence of *H. mustelae*, a gastric species colonizing the stomach of the ferret (Paster *et al.*, 1991; Solnick & Schauer, 2001), was most closely related to that of *H. canis* (75.3% similarity) and its phylogenetic position was unstable, since only a very low bootstrap value (33%) supported its present position in the HSP60 phylogenetic tree. As a result of analyses of the urease α -subunit and 16S rRNA genes of *H. mustelae*, it is suggested that it is phylogenetically more closely related to enterohepatic than to gastric helicobacters (Gueneau & Loiseaux-De Goër, 2002). All evidence from its biology indicates, however, that it is a *Helicobacter* species that primarily colonizes the gastric region (Fox *et al.*, 1990).

Phylogenetic analysis of protein sequences

The same two major clusters (cluster 1 and cluster 2) seen in the DNA sequence tree were formed in the phylogenetic tree constructed from the deduced partial HSP60 protein sequences (180 aa) (Fig. 2). Within cluster 1, the amino acid sequence similarity between species ranged from 94.4 to 99.9% (mean 96.4%). In cluster 2, the overall similarity between protein sequences was 99.4%. Because of the higher similarity between protein sequences than between DNA sequences, none of the species could be differentiated from each other. The high similarity values for HSP60 protein sequences indicate that most of the differences were due to silent mutations. The highest similarity between species (100%) was between *H. troglontum*, *H. bilis* and taxon 8 reference strain (cluster A), while the lowest similarity (84.4%) was between *H. cinaedi* and *H. pullorum*. The partial HSP60 protein sequences for the species *H. pylori*, *H. troglontum* and *H. salomonis* were all identical. Within *H. bizzozeronii*, two of the strains, Emo and R-53, differed from the other three strains by one amino acid, and from each other by two amino acids. *H. felis* strain Kukka differed from the three other strains of the same species by one amino acid.

Specific PCR for differentiating *H. felis*, *H. bizzozeronii* and *H. salomonis*

H. bizzozeronii, *H. felis* and *H. salomonis* are closely related, and phenotypic tests do not differentiate these three species; thus, new genotypic tests are required (Jalava *et al.*, 1997). The primer pair designed for *H. salomonis* (HSALF: 5'-CATTTTCAAAGAGGGCTTGC-3'; HSALR: 5'-GCACACCCCTCAGTTTGTGTT-3') was the only one that showed a species-specific PCR product when tested in the laboratory, producing the 537 bp amplicon only for *H. salomonis* strains. The present PCR method is a useful tool for species-specific identification of *H. salomonis*. The

Fig. 2. Phylogenetic tree based on deduced partial HSP60 protein sequences. Identical sequences are represented as clusters A–F and include the following strains: A, *H. bilis* ATCC 51630^T, KO534B, KO220, KO214, *H. troglontum* HU H95S, taxon 8 CCUG 223435, *H. troglontum* ATCC 700114^T, *H. troglontum* ATCC 43966, *H. troglontum* ATCC 43968, *H. troglontum* ATCC 49310; B, taxon 2 ATCC 49314, taxon 3 ATCC 49320; C, *H. pylori* 7546, *H. pylori* CH-CTX1, *H. pylori* 26695, *H. pylori* CCUG 17874, *H. pylori* Tx30a, *H. pylori* J99; D, *H. salomonis* 06A, *H. felis* Lopki13, *H. felis* DS3, *H. felis* ATCC 49179^T, *H. bizzozeronii* Emo; E, *H. bizzozeronii* CCUG 35545^T, *H. bizzozeronii* 10, *H. bizzozeronii* Yrjälä; F: *H. salomonis* CCUG 37845^T, *H. salomonis* Ko. K. III, *H. salomonis* HS100. The tree was constructed by using the neighbour-joining method and was rooted with sequences from *Campylobacter coli* and *Campylobacter jejuni*. The scale bar represents 0.1 substitutions per site.

intraspecies divergence of the two other species was high and no species-specific primers were detected.

Conclusions

In conclusion, we have shown that the 600 bp HSP60 gene sequence is a suitable phylogenetic marker for the genus *Helicobacter*. The degenerate primers used amplify DNA of several bacterial genera, offering the possibility of extending phylogenetic analysis of diverse bacterial species. New insight into the phylogeny of *Helicobacter* sp. flexispira taxa was achieved, but further studies are needed to describe the taxonomy of taxa 2 and 3 and taxon 8, *H. bilis* and canine flexispiras.

The rather high level of interspecies sequence divergence in the genus *Helicobacter* suggests that development of species-specific PCR or DNA–DNA hybridization tests is also possible for species other than *H. salomonis*.

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