Delineation of two *Helicobacter bilis* genomospecies: implications for systematics and evolution

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The evolution and taxonomy of *Helicobacter bilis* strains isolated in Italy and Finland were studied by phylogenetic analysis of different genes, comparative analysis of small rRNA gene intervening sequence (IVS), amplified fragment length polymorphism analysis and DNA–DNA hybridization. The results of this study divided the *H. bilis* strains into two distinct and divergent genomic groups. In the absence of a specific phenotype or pathotype to distinguish these groups, however, they may be referred to as two genomospecies: *H. bilis sensu stricto* and *Helicobacter* sp. FL56. The phylogenetic network of *gyrB* and *ureB* gene sequences, as well as the comparative analysis of small rRNA gene IVS, suggests independent evolution of the two genomospecies. In particular, *Helicobacter* sp. FL56 seems to be the result of adaptation of an ancestral *H. bilis* strain in a new host. The phenomenon of adaptation to different hosts, or different intestinal niches in the same host, associated with high mutation and recombination rates could explain the evolution and the complex taxonomy of the genus *Helicobacter*. A comprehensive phylogenomics study of this genus would be useful to properly investigate this hypothesis.

Helicobacter bilis, described as a novel species by Fox *et al.* (1995), was originally isolated from mice colonic crypts and caeca, and the bile and livers of mice with hepatitis. Later, it was also isolated from dogs and cats (Eaton *et al.*, 1996; Hänninen *et al.*, 2005; Rossi *et al.*, 2008), rats, hamsters and gerbils (Dewhirst *et al.*, 2000), showing one of the broadest host spectra for this genus. *H. bilis* has cigar-like cells referred to as the ‘Flexispira’ group, which also includes the species *Helicobacter troglodytes* and *Helicobacter aurati* and several taxa, provisionally named *Helicobacter* sp. flexispira, based on 16S rRNA gene sequence analysis (Dewhirst *et al.*,

2000). According to our previous studies, *H. bilis* includes the reference strains of *Helicobacter* sp. flexispira taxon 8 (CCUG 23435, human stool), taxon 2 (ATCC 49314, sheep aborted fetus) and taxon 3 (ATCC 49320, pig stomach aborted fetus) (Hänninen *et al.*, 2005; Hannula & Hänninen, 2007). These studies expanded the host spectrum of the species *Helicobacter bilis* and opened the question of its potential role as a zoonotic agent.

Previously, we described a canine *Helicobacter* strain (FL56) showing 98.5% 16S rRNA gene sequence similarity to the *Helicobacter* sp. flexispira taxon 7 reference strain isolated from a dog stomach (Dewhirst *et al.*, 2000; Hänninen *et al.*, 2005). This strain was indistinguishable from *H. bilis* in terms of cell morphology and biochemical characteristics but had distinct 16S rRNA, *groEL*, *ureB* and *gyrB* gene sequences (Hannula & Hänninen, 2007). Observed sequence divergences did not clearly indicate that this strain belongs to a different species, however, and raised the issue of species delineation for this group of micro-organisms. The aim of this study was to define the taxonomic relationship of *Helicobacter* sp. FL56 and *H. bilis*.

In order to identify strains similar to *Helicobacter* sp. FL56, a collection of 42 Italian *H. bilis* isolates (Rossi *et al.*, 2008) were screened by analysing the *BtsI* restriction patterns of a partial *gyrB* gene. *BtsI* produces a specific pattern for

Abbreviations: AFLP, amplified fragment length polymorphism; IVS, intervening sequence.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *Helicobacter bilis* genomospecies FL56 strains 20/486 and 21/463 are GQ148739 and GQ148740, respectively. The accession numbers for the *gyrB* gene sequences of *Helicobacter bilis sensu stricto* strains 18/522, 21/592, 21/568 and 21/517 are GQ148741, GQ148742, GQ148743 and GQ148744, respectively. The accession numbers for the *ureB* gene sequences of *Helicobacter bilis sensu stricto* strains 18/522, 21/592, 21/568 and 21/517 are GQ148745, GQ148746, GQ148747 and GQ148748, respectively.

Secondary structure of 16S rRNA gene intervening sequences, pairwise alignments of the stem regions of superfamilies I and II, and a phylogenetic network of combined *gyrB* and *ureB* gene sequences of *H. bilis* strains and *Helicobacter* sp. FL56 are available with the online version of this paper.

Helicobacter sp. FL56 partial *gyrB* of about 360 and 200 bp, while no restrictions were predicted for any of the *H. bilis* sequences tested. The forward primer HBILISF and reverse primer HBILISR were used to amplify a 600 bp fragment, as described by Rossi *et al.* (2008). Three microlitres PCR product were digested overnight at 37 °C in a total volume of 20 µl containing 20U *BtsI* (New England Biolabs). The resulting fragments were separated in 1.5% agarose. Five out of 42 Italian *H. bilis* strains, provisionally named as FL56-like *Helicobacter*, showed a *BtsI* restriction pattern identical to the FL56 reference strain.

In order to define the taxonomic position of the Italian FL56-like *Helicobacter* strains, a phylogenetic analysis based on 16S rRNA gene sequences was carried out. The nearly complete 16S rRNA genes of five Italian strains were amplified, sequenced and analysed as described by Rossi *et al.* (2008). In the neighbour-joining tree (Fig. 1), the FL56-like *Helicobacter* strains were interspersed among intestinal *Helicobacter* species and did not clearly cluster in a single clade, as observed for *H. bilis* strains (Hänninen

et al., 2005). The similarity of the 16S rRNA gene sequences among FL56-like Italian strains and the FL56 reference strain varied from 98.6 to 100%; pairwise similarity between FL56-like strains and *H. bilis* and between FL56-like strains and *H. trogonum* ranged from 96.8 to 99% and 95.8 to 97.3%, respectively.

An identical 166 bp intervening sequence (IVS) starting from position 210 (*Escherichia coli* numbering system) corresponding to the V2 area (Neefs *et al.*, 1993) of the 16S rRNA secondary structure, was detected in all FL56 strains. A BLASTN search against the NCBI non-redundant sequence database (nr) revealed a high similarity between the FL56-like IVS and the human *Helicobacter* sp. isolate CCUG 29176 16S rRNA IVS (99.4%; L14634). Similarity was also found with the 16S rRNA IVS of two unclassified *Helicobacter* spp. detected in the gastric mucosa of a harp seal (79%; AY203899) and the faecal samples of capuchine monkeys (68%; AF316888, unpublished). Despite a relatively high similarity observed among 16S rRNA gene sequences of FL56-like and *H. bilis* strains, the IVS found

Fig. 1. Unrooted tree, based on 16S rRNA partial gene sequences (1269 bp), showing the phylogenetic relationships of the five Italian FL56-like *Helicobacter* strains together with the other species of the genus *Helicobacter*. *H. bilis* Hb1^T=ATCC 51630^T (Fox *et al.*, 1995); *Helicobacter* sp. taxon 7 Dog-4 = *Helicobacter* sp. taxon 7 1302 (Eaton *et al.*, 1996); *H. bilis* Eaton 1502 = *H. bilis* 1502 (Eaton *et al.*, 1996). Bar, 0.005 nt substitutions per base. Numbers at the nodes indicate support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps).

in both bacterial groups were extremely divergent, as previously observed (Hänninen *et al.*, 2005). A comparison of the secondary structure among the available 16S rRNA IVS of different *Helicobacter* species was carried out to further examine the distribution and interrelationships of these insertion elements. 16S rRNA gene sequences were obtained from GenBank and we considered only those sequences of well characterized strains belonging to species of the genus *Helicobacter* with validly published names (<http://www.bacterio.cict.fr/h/helicobacter.html>). The rRNA gene structure was predicted by free-energy minimization using the mfold program (Zuker, 2003).

In addition to *H. bilis* and FL56-like strains, the species presenting IVS in the V2 portion of the small rRNA gene were *Helicobacter canis*, *H. cinaedi*, *H. fennelliae*, *H. typhlonius* and *H. mastomyrinus*. The highest level of intraspecific sequence diversity was observed among *H. bilis* strains (94.5%) while the sequence identity among different strains of the other species was almost 100%. All sequences possess an imperfect terminal inverted repeat of about 18–32 bases that form the primary stem in the secondary structure; the rest of the sequence folds into secondary stems and loops. As described by Pabbaraju & Sanderson (2000), IVS were grouped based on sequence similarity. Briefly, the insertion sequences showing more than 90% nucleotide identity were placed in different sequence families. Those IVS families which had nearly identical nucleotide sequences in the stem region were grouped into IVS superfamilies. Seven sequence families corresponding to the six species with validly published names and FL56 were identified. The different sequence families were then grouped into five superfamilies (SF). SF I comprises the insertion sequences found in *H. bilis* and *H. cinaedi* strains, SF II those of *H. canis* and FL56 strains, while each of the other superfamilies contain only one sequence family. The secondary structures of the different families belonging to SF I and SF II are shown in Supplementary Fig. S1(a) and (b) (available in IJSEM Online) and the alignments of the stem regions in Supplementary Fig. S2.

Phylogenetic relationships of FL56-like and *H. bilis* strains were further examined by analysis of *gyrB* and *ureB* genes. Partial *gyrB* genes of the five Italian FL56-like *Helicobacter* strains and four randomly selected Italian *H. bilis* strains were amplified by using the primers HBILISF and HBILISR and directly sequenced (Rossi *et al.*, 2008). Partial *ureB* gene sequences of the strains were obtained as described previously (Hänninen *et al.*, 2005). Sequences were aligned in MEGA4 by CLUSTAL W and distance matrices were calculated using the model of Jukes and Cantor (Tamura *et al.*, 2007). The FL56 reference strain and all Italian FL56-like *Helicobacter* strains showed identical partial *gyrB* and *ureB* nucleotide sequences. The *gyrB* sequence similarity between *Helicobacter* sp. FL56 and the *H. bilis* strains varied from 94.7 to 95.1%. Likewise, pairwise similarities of the *ureB* sequence of FL56 and *H. bilis* ranged from 92.2 to 93.2%. The overall *gyrB* and *ureB* sequence similarity among the *H. bilis* strains was equal to or greater than 95.8 and 97.9%, respectively.

In order to analyse for recombination events, we performed a priori tree-independent analysis using neighbor-net (Bryant & Moulton, 2004). The *gyrB* and *ureB* nucleotide sequences were combined and the neighbor-net method was applied to reconstruct a phylogenetic network using SplitsTree4 v4.10 (Huson & Bryant, 2006). The phylogenetic network is shown in Supplementary Fig. S3. A long branch separated FL56 from the *H. bilis* strains, which are linked by an interconnected network suggesting recombination events among the strains.

Although the sequence data suggested that the FL56-like strains could represent a coherent taxon, the incongruence of the 16S rRNA gene phylogeny and the relatively low *gyrB* and *ureB* sequence distance observed between FL56 and *H. bilis* did not support this hypothesis. For that reason, amplified fragment length polymorphism analysis (AFLP) and DNA–DNA hybridization were performed to further examine the relationships between these two taxa.

AFLP reactions were carried out as described by Debruyne *et al.* (2009). Similarity between normalized profiles was

Fig. 2. Dendrogram showing UPGMA cluster analysis of AFLP profiles of 15 strains representing species of the genus *Helicobacter*.

determined by the Pearson product moment correlation coefficient and an unweighted pair group method with arithmetic mean (UPGMA) dendrogram was reconstructed. The results of the numerical comparison of AFLP profiles of 15 different strains, representing different species of the genus *Helicobacter*, are shown in a dendrogram (Fig. 2). UPGMA analyses indicated five distinct phenons at the 24.1 % genetic similarity level, of which four represented species with validly published names: *H. aurati*, *H. muridarum*, *H. bilis* and *H. trogontum*. The remaining phenon comprised the *Helicobacter* sp. FL56 strains that clustered together at 68.1 %. The *H. bilis* strains, including the reference strains of taxons 2, 3 and 8, formed a distinct clade at the 35.3 % similarity level. Likewise, all the reference strains of *H. trogontum* taxons 1, 4 and 5, as well as the type strain, clustered together at the 45.6 % similarity level.

DNA–DNA hybridization was performed at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) between *Helicobacter* sp. ‘flexispira’ strain FL56 and *H. bilis* ATCC 51630^T. For this purpose, DNA was isolated by using a French pressure cell (Thermo Spectronic) and the hybridization was performed twice as described by De Ley *et al.* (1970) and Huß *et al.* (1983). Quantitative DNA–DNA hybridization between the FL56 reference strain and *H. bilis* ATCC 51630^T showed a 56.0/60.9 % hybridization rate at 62 °C.

The sequence data, AFLP results and DNA–DNA hybridization clearly indicated that the FL56-like strains belong to a coherent taxon separate from *H. bilis*. The phenotypic data did not justify, however, the description of a novel species. Although extensive genetic divergence was observed, these two taxa were indistinguishable in terms of biochemical profile, ultrastructural morphology and whole-cell protein profile (Hannula & Hänninen, 2007; Rossi *et al.*, 2008). Moreover, both taxa were isolated from a canine host and no differences in pathogenicity were observed (Rossi *et al.*, 2008). In the absence of a specific phenotype or pathotype, *H. bilis* can be regarded as a ‘complex’ species comprising at least two genomospecies, *H. bilis sensu stricto* and *Helicobacter* sp. FL56, using the criteria suggested by Wayne *et al.* (1987) and Gevers *et al.* (2006). AFLP profile analysis, phylogenetic analysis of *gyrB* and *ureB* gene sequences and the *BtsI* restriction pattern of *gyrB* can be used to differentiate the *H. bilis* genomospecies.

Whole genome fingerprinting by AFLP is a powerful tool for taxonomic and subtyping analyses of various bacterial species (Duim *et al.*, 2001; On *et al.*, 2003). As observed for *Campylobacter concisus* and *Campylobacter lari* (Aabenhus *et al.*, 2005; Debruyne *et al.*, 2009), our results suggest that AFLP analysis has the potential to differentiate *H. bilis* genomospecies and in general to be suitable for the analysis of the complex taxonomy of the different taxa belonging to the ‘Flexispira’ morphology group. Despite the discrepancy between the phylogeny of ribosomal and protein coding genes observed by Dewhirst *et al.* (2005), the results of the

present study confirm our previous conclusions (Hänninen *et al.*, 2003, 2005) and provide evidence to understand the taxonomy of this group of micro-organisms.

The phylogenetic network of *gyrB* and *ureB* gene sequences revealed a process of recombination among *H. bilis sensu stricto* strains but not between the two genomospecies, suggesting the two lineages have evolved independently. This hypothesis is supported by the fact that the two genomospecies carry a different insertion sequence in the V2 area of the small rRNA gene. In contrast to what is observed for the large rRNA gene IVS in the genera *Campylobacter* (Meinersmann *et al.*, 2009) and *Enterobacteriaceae* (Pronk & Sanderson, 2001), the process of acquisition of insertion sequences in the 16S rRNA gene of species of the genus *Helicobacter* is more likely due to vertical transmission or intraspecies lateral transfer than to interspecies recombination. Indeed, each *Helicobacter* species (or genomospecies) presenting IVS in the 16S rRNA gene harbours a unique sequence. These results appear to be in contrast with the hypothesis of Dewhirst *et al.* (2005) that helicobacter 16S rRNA gene sequences have been modified by horizontal exchange, but indicate genetic isolation (Matic *et al.*, 1996) of these *Helicobacter* species. However, similarities in the stem region of IVS secondary structure found in two cases (*H. bilis sensu stricto* and *H. cinaedi*; FL56 and *H. canis*) could support lateral gene transfer among different species of the genus *Helicobacter*. The presence of IVS belonging to the same superfamily may indicate that *H. bilis sensu stricto* and *H. cinaedi*, as well as FL56 and *H. canis*, had a common ancestral source of the IVS. Neither *H. cinaedi* nor *H. canis* are phylogenetically (Hannula & Hänninen, 2007) or phenotypically (Totten *et al.*, 1985; Stanley *et al.*, 1993) related with ‘Flexispira’ *Helicobacter* species (*H. bilis*, *H. trogontum* and *H. aurati*) but they have frequently been isolated from the same hosts (Kiehlbauch *et al.*, 1995; Rossi *et al.*, 2008). Moreover, using multiple culture-independent methods, Recordati *et al.* (2009) showed that *H. bilis*, *H. cinaedi* and *H. canis* have the same regional distribution (caecum and colon) and mucosal localization (mucosal surface and within the crypts) in the gastrointestinal tract of dogs. The cohabitation of these species in the same niche could be at the origin of the lateral transfer of ancestral IVS. However, except for the stem region, the insertion sequences do not show significant similarity, suggesting that even though these species shared the same niches and hosts, they remained substantially isolated. The genetic isolation, as the basis of the apparent species-specificity of helicobacter 16S rRNA gene IVS, could explain the independent evolution of the two *H. bilis* genomospecies.

Therefore, *Helicobacter* sp. FL56 genomospecies appears to be a distinct *Helicobacter* lineage characterized by a clonal structure and could be the result of adaptation and coevolution of an ancestral *H. bilis* strain in a new host. Coevolution between *Helicobacter pylori* and its human host is well described (Linz *et al.*, 2007) and a similar event could be hypothesized among other *Helicobacter* species

and their hosts as well (Linz & Schuster, 2007). It is, however, evident that some species, like *H. bilis*, are able to colonize different hosts (Fox *et al.*, 1998; Matsukura *et al.*, 2002; Rossi *et al.*, 2008) or different niches of the same host (Eaton *et al.*, 1996; Fox *et al.*, 1998; Matsukura *et al.*, 2002), showing a high flexibility of adaptation to different environments. Adaptation, in association with high mutation and recombination rates, has recently been described for a host-jump from early humans to large felines resulting in *Helicobacter acinonychis* (Eppinger *et al.*, 2006). Host-jump followed by host-adaptation could explain the evolution and the complex taxonomy of the genus *Helicobacter* and the increasing number of newly described species. A comprehensive phylogenomics study of the genus *Helicobacter* would be useful to properly investigate this hypothesis.

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