NOTE**Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and applied implications**Clare S. Harrington^{1,2} and Stephen L. W. On²Author for correspondence: Clare S. Harrington. Tel: +45 35 30 02 83. Fax: +45 35 30 01 20.
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Phylogenetic relationships of *Campylobacter hyointestinalis* subspecies were examined by means of 16S rRNA gene sequencing. Sequence similarities among *C. hyointestinalis* subsp. *lawsonii* strains exceeded 99.0%, but values among *C. hyointestinalis* subsp. *hyointestinalis* strains ranged from 96.4 to 100%. Sequence similarities between strains representing the two different subspecies ranged from 95.7 to 99.0%. An intervening sequence was identified in certain of the *C. hyointestinalis* subsp. *lawsonii* strains. *C. hyointestinalis* strains occupied two distinct branches in a phylogenetic analysis of the genus *Campylobacter*, emphasizing the need for multiple strain analysis when using 16S rRNA gene sequence comparisons for taxonomic investigations.

Keywords: *Campylobacter hyointestinalis*, 16S rRNA, sequence diversity, phylogeny, taxonomy

Campylobacter hyointestinalis was first described from pigs with enteric disease (Gebhart *et al.*, 1983), and has since been isolated from cases of enteritis in other animals and humans (Ursing *et al.*, 1984; Gebhart *et al.*, 1985; Edmonds *et al.*, 1987; Hill *et al.*, 1987). Recent studies determined that some strains isolated from the stomachs of pigs were distinct from the enteric isolates, and the species description of *C. hyointestinalis* was subsequently emended to recognize two subspecies (On *et al.*, 1995); *C. hyointestinalis* subsp. *hyointestinalis* (principally of enteric origin), and *C. hyointestinalis* subsp. *lawsonii* (mainly associated with pig stomachs).

Comparative analysis of 16S rRNA gene sequences is a frequently used means of determining the phylogenetic position of a given taxon (Murray *et al.*, 1990; Stackebrandt & Goebel, 1994). However, recent studies have suggested that multiple strains should be investigated to evaluate the degree of sequence diversity within and between species (Clayton *et al.*, 1995). We present here an analysis describing the phylogenetic position of *C. hyointestinalis* subsp. *lawsonii*, and the extent of 16S rRNA gene sequence diversity within and between the two *C. hyointestinalis* subspecies.

Type, field and reference strains used in this study are listed in Table 1, together with their sources. All strains were cultured under microaerobic conditions on 5% (v/v) horse blood agar for 2–3 d at 37 °C. The seven field isolates were identified by computer-assisted analysis of 55–57 standardized phenotypic tests (On *et al.*, 1996) and, further, by analysis of *Sma*I restriction profiles (On & Vandamme, 1997). Six strains were phenotypically identified as *C. hyointestinalis* subsp. *hyointestinalis*, with identification scores ranging from 89.6 to 100% (mean 97.4%). SVS 3035 was not identified by this method, but differed from isolates SVS 3038 and 3050 in only three tests; alkaline phosphatase production (weak positive), resistance to 5-fluorouracil (positive), and hydrogen sulphide production in triple-sugar iron agar (negative). *Sma*I-derived macrorestriction profiles (not shown) for all field strains, including SVS 3035, were characteristic of *C. hyointestinalis* subsp. *hyointestinalis*, according to previously defined criteria (On & Vandamme, 1997). All isolates possessed the two fragments (48.5–60 kb and ~204 kb) previously found to be characteristic for *C. hyointestinalis* subsp. *hyointestinalis*, whereas the two fragments (242–254 kb and >550 kb) characteristic for *C. hyointestinalis* subsp. *lawsonii* were absent. This identification is consistent with their faecal origin.

Primers and methods used for DNA extraction, PCR amplification, and direct sequencing of 16S rRNA

The GenBank accession numbers for the 16S rRNA gene sequences determined in this paper are AF097681–AF097691.

Table 1. Strains, sources and sequence accession numbers

Taxon*	Strain†	Clinical and geographical origin (if known)	GenBank no.‡
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	NCTC 11608 ^T	Porcine intestine, USA	AF097689
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SVS 3035	Porcine faeces, Denmark	AF097690
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SVS 3038	Porcine faeces, Denmark	AF097691
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SVS 3050	Porcine faeces, Denmark	(AF097690)
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SVS 3056	Porcine faeces, Denmark	(AF097690)
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CCUG 27631	Porcine stomach, Sweden	AF097683
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CHY 4	Porcine stomach, England	AF097684
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CHY 5 ^T	Porcine stomach, England	AF097685
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CHY 6	Porcine stomach, England	AF097686
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CHY 7	Porcine stomach, England	AF097687
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CHY 8	Porcine stomach, England	AF097688
<i>C. hyointestinalis</i> subsp. <i>lawsonii</i>	CHY 9	Porcine stomach, England	(AF097688)
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SCRL 0425	Bovine diarrhoea, Scotland	AF097681
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SCRL 0469	Bovine diarrhoea, Scotland	(AF097682)
<i>C. hyointestinalis</i> subsp. <i>hyointestinalis</i>	SCRL 0943	Bovine diarrhoea, Scotland	AF097682
<i>Arcobacter nitrofigilis</i>	CCUG 15893 ^T	Roots of <i>Spartina alterniflora</i> , Canada	L14627
[<i>Bacteroides</i>] <i>ureolyticus</i> §	ATCC 33837 ^T	Human amniotic fluid, Canada	L04321
<i>Campylobacter coli</i>	CCUG 11283 ^T	Porcine faeces, Belgium	L04312
<i>Campylobacter concisus</i>	ATCC 33237 ^T	Human gingival sulcus, USA	L04322
<i>Campylobacter curvus</i>	ATCC 35224 ^T	Human alveolar abscess, USA	L04313
<i>Campylobacter fetus</i> subsp. <i>fetus</i>	ATCC 27374 ^T	Ovine foetus brain, France	L04314
<i>Campylobacter fetus</i> subsp. <i>venerealis</i>	ATCC 19438 ^T	Bovine vaginal mucus, England	L14633
<i>Campylobacter gracilis</i>	ATCC 33236 ^T	Human periodontitis, USA	L04320
<i>Campylobacter helveticus</i>	NCTC 12470 ^T	Feline faeces, Switzerland	U03022
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i>	CCUG 11284 ^T	Bovine faeces, Belgium	L04315
<i>Campylobacter jejuni</i> subsp. <i>doylei</i>	CCUG 24567 ^T	Human infantile diarrhoea, Australia	L14630
<i>Campylobacter lari</i>	CCUG 23947 ^T	Herring gull cloacal swab, UK	L04316
<i>Campylobacter lari</i> biovar 'UPTC'	CCUG 18267	River water, UK	L14631
<i>Campylobacter mucosalis</i>	CCUG 6822 ^T	Porcine small intestine, UK	L06978
<i>Campylobacter rectus</i>	ATCC 33238 ^T	Human periodontal pocket, USA	L04317
<i>Campylobacter showae</i>	ATCC 51146 ^T	Human gingival crevice, Japan	L06974
<i>Campylobacter sputorum</i> biovar <i>sputorum</i>	LMG 7795 ^T	Human oral cavity, USA	X67775
<i>Campylobacter</i> sp. Pig	PGC 40-6AT	Porcine stomach, USA	L04318
<i>Campylobacter upsaliensis</i>	CCUG 14913 ^T	Canine faeces, Sweden	L14628
<i>Helicobacter pylori</i>	ATCC 43504 ^T	Human gastric antrum gastritis, Australia	M88157

* All *C. hyointestinalis* strains were identified to subspecies level by phenotypic and genotypic methods as described in the text.

† ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection of the University of Göteborg, Göteborg, Sweden; CHY, *Campylobacter hyointestinalis*-like; LMG, culture collection of the Laboratorium voor Microbiologie, University of Ghent, Belgium; NCTC, National Collection of Type Cultures, London, UK; PGC, The Proctor and Gamble Co., Cincinnati, OH, USA; SCRL, Scottish *Campylobacter* Reference Laboratory, Aberdeen, UK; SVS, Statens Veterinære Serumlaboratorium, Copenhagen, Denmark; ^T, type strain.

‡ 16S rRNA gene sequences available for electronic retrieval from the GenBank database under the indicated accession numbers. *C. hyointestinalis* strains sharing identical sequences are indicated by parentheses around the accession number given for the representative strain from which the sequence was actually submitted.

§ Generically misnamed. This species is not a true *Bacteroides*, but belongs in rRNA superfamily VI (Vandamme *et al.*, 1995).

genes were as described previously (On *et al.*, 1998), but with the following modifications: primer 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T), representing nucleotides 1492–1513 of the *Escherichia coli* 16S rRNA gene (Brosius *et al.*, 1978), was used in place of primer 1392r for the initial PCR reaction with primer 27f (AGA GTT TGA TCM TGG CTC AG) and for subsequent sequencing of the PCR product. Centricon C-100 columns (Amicon) were used in place

of QIAquick (QIAGEN) kits for purification of PCR products prior to direct sequencing. An additional primer (CHY 4-IVSr; 5'-GCC GAG AGC TCT TAA TAA TAC) was used to sequence the reverse strand of strain CHY 4 across its intervening sequence. The program RNA was used for sequence alignment and phylogenetic analysis, using secondary structure to aid alignment of less conserved regions, as described previously (Paster & Dewhirst, 1988). RNA is a program

Fig. 1. Phylogenetic tree for 15 *C. hyointestinalis* strains and for 20 reference *Campylobacter*, *Arcobacter* and *Helicobacter* strains, based on 16S rRNA gene sequence similarity. The scale bar represents 5% difference in nucleotide sequence, as determined by measuring the lengths of the horizontal lines connecting any two strains. ^T, Type strain.

for analysis of 16S rRNA data written in Microsoft QuickBASIC for use on IBM-compatible computers, and was a generous gift of Floyd E. Dewhirst (Forsyth Dental Centre, Boston, USA). *C. hyointestinalis* sequences were aligned with a pre-aligned database of ~70 *Campylobacter*, *Arcobacter*, *Helicobacter* and *Wolinella* reference strain sequences, of which 20 were included in the final phylogenetic analysis (Table 1). Distances were calculated using only those base positions for which 90% of the strains had data (1393 positions). Unknown bases or alignment gaps opposite these positions were further eliminated from analysis on a pairwise basis. Distances were corrected for multiple base changes by the method of Jukes & Cantor (1969). The phylogenetic tree shown in Fig. 1 was constructed using the neighbour-joining method of Saitou & Nei (1987).

The expected (~1500 bp) amplicon was obtained for all strains except for CHY 4 and CHY 5. CHY 4 yielded an enlarged product of ~1750 bp, whereas CHY 5 yielded both the expected and the enlarged sizes of amplicon, only the smaller of which was sequenced. Sequences were obtained (both strands) over positions 38–1486 (*E. coli* numbering; Brosius *et al.*, 1978), giving lengths of 1418–1423 bp, except for CHY 4, for which an increased length of 1662 bp was obtained. The increased length was due to the presence

of an intervening sequence (IVS) of 244 bp, located after base 215 (*E. coli* numbering). Intervening sequences of diverse lengths and sequences have been described previously at this location in the 16S rRNA gene for several *Campylobacter* species, including *Campylobacter sputorum*, *Campylobacter helveticus*, *Campylobacter rectus* and *Campylobacter curvus* (listed by Etoh *et al.*, 1998). Distribution of intervening sequences in the 16S rRNA genes of *C. hyointestinalis* resembles that of *C. helveticus* (Linton *et al.*, 1994) and *C. rectus* (Etoh *et al.*, 1998), in that a given strain may possess the enlarged gene (as for CHY4), both types of gene (CHY 5), or the non-IVS-containing gene alone (all other strains).

Eleven different 16S rRNA gene sequences were obtained from 15 *C. hyointestinalis* strains (Table 1). Comparison of the aligned sequences, including PGC 40-6AT, which was identified as *C. hyointestinalis* subsp. *lawsonii* (see below), identified 81/1423 (5.7%) polymorphic sites, as summarized in Fig. 2. The majority of these sites represented sequence differences found between the two subspecies, most noticeably in a 24 base hypervariable region from base 1379 to 1402 of the submitted NCTC 11608 sequence (Fig. 2). *C. hyointestinalis* subsp. *hyointestinalis* SVS 3038 presented an anomaly in that its base composition at the variable sites resembled that exhibited by *C. hyointestinalis* subsp. *lawsonii*.

Linton *et al.* (1996) have described a species-specific PCR test for *C. hyointestinalis*. The forward (CFCH57F) and reverse (CH1344R) primers described correspond to base positions 20–39 and 1267–1286, respectively, of our submitted sequence for NCTC 11608^T. Examination of Fig. 2 identifies three polymorphic sites within the 3'-region of the annealing site for the forward primer (positions 34, 35 and 39). These differences are found in three of the five sequences representing *C. hyointestinalis* subsp. *hyointestinalis*, and in all *C. hyointestinalis* subsp. *lawsonii* strains (not formally described at the time of the study by Linton *et al.*, 1996). It is feasible that the specificity of this PCR test may be compromised by the existence of such polymorphic sites.

Phylogenetic analysis of the sequences delineated *C. hyointestinalis* strains into two principal groups within the genus *Campylobacter* (Fig. 1). All but one of the *C. hyointestinalis* subsp. *hyointestinalis* strains, including the type strain, were related at similarity levels ranging between 98.9% (NCTC 11608^T with SCRL strains) and 100% (SCRL 0469 and 0493). These seven strains fell into a loose clade, comprising two branches related at ~99.3%. The remaining *C. hyointestinalis* subsp. *hyointestinalis* strain (SVS 3038) fell as an outlier (98.7% mean similarity) to the second main group, comprising all seven *C. hyointestinalis* subsp. *lawsonii* strains, together with a previously unidentified *Campylobacter* strain (*Campylobacter* sp. Pig strain PGC 40-6AT). Interstrain similarity levels within this latter group ranged between 99.0% (PGC 40-6AT

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3334456679000013556888234440011335566615886678900104466677788348013901788889999999
45901501183457906996781338587803560457317288881885372391461612102567491268246902
NCTC 11608 GTAAGTACCGACATGTCATTGTATAGCGGGCCCCACTGTGGCTAGCGCGAATATATTAAATGCCGACGTATGGGTACCCTCC
0425 AAGTAAGA.....CC...GC.....TGTC...T.....
0943 AAGTAAGA.....C...GCT.....TGTC...T.....
SVS 3035 .....A.....TGTC...T...T.....
SVS 3038 AAGTAAGAT.....GCC...G....CA.ATGTGTCA.A.T..AT....GCGGACGTACAGTT....CCAACCGTTGT
CCUG 27631 AAGTAAGA.....GCC.....CATATGTGTACAAA.CCCGTGCGTGCGGACGTACAG.T...CGCCAACCGTTGT
CHY 4 AAGTAAGA...TG....C.CA.....ATATGTGTACAAA.CCCGTGCG.GCGGACGTACAGTT....CCAACCGTTGT
CHY 5 AAGTAAGA.....GCC.....ATAT.TGTACAAA.CCCGTGCGGCGGACGTACAGTT....NCCAACCGTTGT
CHY 6 AAGTAAGA..NNNNCN.C...C...CGCA.ATGTGTACAAA.C.CGTGCG.GCGGACGTACAGTT....CCAACCGTTGT
CHY 7 AAGTAAGA..G..ACG.C.C...C...CA.ATGTGTACAAA.CCCGTGCG.GCGGACGTACAGTT...CGCCAACCGTTGT
CHY 8 AAGTAAGA.....GCC.....CA.ATGTGTACAAA.C.CGTGCG.GCGGACGTACAGTT...CGCCAACCGTTGT
PGC 40-6AT AAGTAAGA.....GC.....CGCA.ATGTGTACAAA.CCCGTGCG.GCGGACGTACAGTTGA...CCAACCGTTGT

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Fig. 2. Alignment of all *C. hyointestinalis* 16S rRNA gene sequences with the sequence for NCTC 11608^T, showing only polymorphic sites. The numbers (in vertical format) above the sequences correspond to the base positions of the submitted NCTC 11608^T sequence. Bases identical to the NCTC 11608^T sequence are represented by a full stop (.)

with CHY 7) and 100% (CHY 8 with CHY 9), with 99.4% mean similarity. We therefore consider PGC 40-6AT to be *C. hyointestinalis* subsp. *lawsonii*, which is consistent with its porcine gastric origin (cited by Eaton *et al.*, 1993), since most *C. hyointestinalis* subsp. *lawsonii* strains described to date have been isolated from pig stomachs. The taxonomic position of SVS 3038 is noteworthy, since it demonstrates a clear phylogenetic affinity with *C. hyointestinalis* subsp. *lawsonii* strains, albeit as an outlier. However, its porcine enteric origin, phenotype and macrorestriction profile are all wholly typical of *C. hyointestinalis* subsp. *hyointestinalis*.

It is generally accepted that DNA-DNA hybridization is superior to 16S rRNA phylogenetic analysis in delineating relationships between closely related taxa (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). At 16S rRNA similarities of 97.5% and above, DNA reassociation values can either be significantly lower than 70%, or may be as high as 100% (Fox *et al.*, 1992; Stackebrandt & Goebel, 1994). Conversely, high levels of intraspecific sequence variation have been identified by Clayton *et al.* (1995), and also in the current study, where we describe intraspecific sequence similarities of as low as 95.7% for *C. hyointestinalis*. Current guidelines suggest that 97.0% is the threshold 16S rRNA similarity value below which two organisms may be considered to represent distinct species (Stackebrandt & Goebel, 1994). However, these authors also recommended that this threshold value be lowered so as to maintain flexibility in the phylogenetic definition of a species by parameters of DNA reassociation. Certain of our data support this recommendation. Previous experiments using a well-established DNA renaturation method (De Ley *et al.*, 1970) showed that the type strains of the two *C. hyointestinalis* subspecies (NCTC 11608 and CHY 5) were 71% related by DNA-DNA hybridization (On *et al.*, 1995), despite sharing just 96.1% 16S rRNA sequence similarity (this study). It could be argued that each subspecies deserves elevation to distinct species status,

since DNA hybridization values of down to 56 % were detected between other strains of the two subspecies (On *et al.*, 1995). However, the overall phenotypic, ecologic and genotypic consistency between *C. hyointestinalis* subsp. *hyointestinalis* and *C. hyointestinalis* subsp. *lawsonii* (On *et al.*, 1993, 1995; On & Vandamme, 1997), in addition to the phylogenetic position of SVS 3038 (this study), favour the continued delineation of these taxa as subspecies. It is clear from this work that the polyphasic taxonomic approach remains essential for the accurate classification and identification of bacteria (Murray *et al.*, 1990; Stackebrandt & Goebel, 1994; Vandamme *et al.*, 1996). We can only endorse its continued practice.

Acknowledgements

We are indebted to Enevold Falsen (Culture Collection of the University of Göteborg, Sweden) and to Fiona Thomson-Carter (Scottish Campylobacter Reference Laboratory) for their invaluable support by provision of reference and field strains, respectively. C.S.H. is indebted to the European Molecular Biology Organization (EMBO, Germany) for a short-term International Fellowship award, and to the Wellcome Trust (London, UK) for an International Travel Grant. We thank Floyd Dewhirst (Forsyth Dental Centre, Boston, USA) for providing the computer program RNA. C.S.H. further thanks him for his very helpful discussions concerning 16S rRNA sequence alignment. Thanks are also due to Kenny Reay and Phil Carter (University of Aberdeen) for their expert technical assistance with running sequencing reactions, and to Penny Jordan (Danish Veterinary Laboratory) for technical assistance with electrophoretic and phenotypic methods.

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