Two types of 16S rRNA gene are found in *Campylobacter helveticus*: analysis, applications and characterization of the intervening sequence found in some strains

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In the recently described species *Campylobacter helveticus*, two sizes of PCR amplicon were detected with primers homologous to conserved regions of the 16S rRNA gene. A conventionally sized gene was sequenced from the type strain, NCTC 12470, placing the new species as phylogenetically related to *C. upsaliensis* and the thermotolerant campylobacters. This nucleotide sequence enabled PCR primers to be designed for use in rapid molecular identification of *C. helveticus* and its closest phylogenetic relative, *C. upsaliensis*. When this assay was employed to characterize 22 '*C. upsaliensis*-like' isolates, twelve were identified as *C. helveticus* and nine as *C. upsaliensis*, in agreement with data obtained with a *C. helveticus*-specific DNA probe. A 550 bp amplicon internal to the 16S rRNA gene of *C. helveticus* was used to determine restriction fragment length polymorphisms (RFLPs) in genomic Southern blots, confirming that the copy number of the *C. helveticus* gene was three, and identifying nine 16S rRNA gene profiles. In 5/12 *C. helveticus* isolates identified by PCR, an enlarged amplicon was detected. The enlarged 16S rRNA gene of one of these strains, NCTC 12838, was sequenced and shown to contain an atypical intervening sequence (IVS) of 148 nucleotides. The position and size of such an IVS was inferred in the other four isolates by PCR with primers 5' and 3' to its position in NCTC 12838. This is a first report of an IVS in the 16S rRNA gene of a eubacterium.

Keywords: *Campylobacter helveticus*, 16S rRNA gene, intervening sequence, phylogenetic analysis, molecular subtyping

INTRODUCTION

Campylobacter helveticus is a recently described member of the genus *Campylobacter* (Stanley *et al.*, 1992) isolated from domestic cats. It is important to establish precise means for identification and subtyping of newly described *Campylobacter* in order to clarify their role in animal or human disease.

The gene encoding 16S ribosomal RNA has been

sequenced from *Escherichia coli* and numerous other bacterial species. Its length is generally uniform, whilst variable regions within the gene have been employed to derive phylogenetic relationships between bacterial species (Woese, 1987).

In the study described here, by analysis of the nucleotide sequence of the conventionally sized 16S rRNA gene found in the type strain of *C. helveticus* NCTC 12470^T, we have established the phylogenetic position of the species, designed a specific PCR-based identification method, and described a molecular subtyping scheme for isolates of this species. Certain strains of *C. helveticus* contain atypically large 16S rRNA genes, and we have characterized the enlarged gene from one such strain, demonstrating the presence of an intervening sequence, whose

Abbreviations: IVS, intervening sequence; RFLP, restriction fragment length polymorphism.

The GenBank accession number for the 16S RNA sequence of *C. helveticus* NCTC 12470^T is U03022.

Table 1. Bacterial strains

Strain	Reference‡	Source	Selenite reduction	pCH1 hybridization	PCR identification		16S gene profile
					CHCU§	CH	
<i>Campylobacter helveticus</i> *							
NCTC 12470 ^T	1	Feline, faecal	—	+	+	+	<i>Cb</i> -RI
NCTC 12471	1		—	+	+	+	<i>Cb</i> -RVII
NCTC 12472	1		—	+	+	+	<i>Cb</i> -RIV
NCTC 12473	1		—	+	+	+	<i>Cb</i> -RI
A722-91	1		—	+	+	+	<i>Cb</i> -RI
A723-91	1		—	+	+	+	<i>Cb</i> -RI
NCTC 12847	1		—	+	+	+	<i>Cb</i> -RVI
A725-91	1		—	+	+	+	<i>Cb</i> -RI
A727-91	1	Canine, faecal	—	+	+	+	<i>Cb</i> -RI
E504-90	2		—	+	+	+	<i>Cb</i> -RI
E1797-91	2		—	+	+	+	<i>Cb</i> -RI
NCTC 12845	2		—	+	+	+	<i>Cb</i> -RII
E1997-91	2		—	+	+	+	<i>Cb</i> -RI
E2018-91	2		—	+	+	+	<i>Cb</i> -RII
E2032-91	2		—	+	+	+	<i>Cb</i> -RI
NCTC 12848	2		Feline, faecal	—	+	+	+
E2205-91	2	Feline, faecal	—	+	+	+	<i>Cb</i> -RI
E2220-91	2		—	+	+	+	<i>Cb</i> -RI
E2289-91	2		—	+	+	+	<i>Cb</i> -RI
E2388-91	2		—	+	+	+	<i>Cb</i> -RI
<i>Campylobacter upsaliensis</i>							
NCTC 11541 ^T	3	Canine, faecal	+	—	+	—	NT
' <i>C. upsaliensis</i> -like' strains†							
C121-92	4	Feline, faecal	—	+	+	+	<i>Cb</i> -RI
C123-92	4	Canine, faecal	+(w)	—	+	—	NT
C126-92	4	Canine, faecal	+	—	+	—	NT
NCTC 12846	4	Feline, faecal	—	+	+	+	<i>Cb</i> -RV
C128-92	4	Feline, faecal	+(w)	—	+	—	NT
C129-92	4	Canine, faecal	+	—	—	—	NT
C130-92	4	Feline, faecal	—	+	+	+	<i>Cb</i> -RI
C131-92	4		+	—	+	—	NT
C132-92	4		—	+	+	+	<i>Cb</i> -RI
C133-92	4		—	+	+	+	<i>Cb</i> -RI
C135-92	4		—	+	+	+	<i>Ch</i> -RI
C136-92	4		+(w)	—	+	—	NT
C137-92	4		—	—	+	—	NT
C138-92	4		—	+	+	+	<i>Cb</i> -RII
NCTC 12849	4		—	+	+	+	<i>Cb</i> -RIX
C140-92	4		—	+	+	+	<i>Cb</i> -RIII
C141-92	4		—	—	+	—	NT
C142-92	4		+	—	+	—	NT
C143-92	4		—	+	+	+	<i>Cb</i> -RI
C144-92	4		—	+	+	+	<i>Cb</i> -RIII
C145-92	4		+(w)	—	+	—	NT
NCTC 12838	4		—	+	+	+	<i>Cb</i> -RIII

(w), weak reaction; NT, not tested; superscript T denotes type strain.

* All the *C. helveticus* strains originated from Switzerland.† All the '*C. upsaliensis*-like' strains originated from the UK.‡ 1, Stanley *et al.* (1992); 2, this report; 3, Sanstedt *et al.* (1983); 4, strains received from P. Griffith (University of Reading) as *C. upsaliensis*.

§ CHCU, band of ~ 1260 or ~ 1410 bp or two bands of ~ 1260 and ~ 1410 bp.

|| CH, band of ~ 830 or ~ 960 bp, or two bands of ~ 830 and ~ 960 bp.

nucleotide sequence and potential secondary structure we have determined.

METHODS

Bacterial strains, culture conditions and phenotypic characterization. *Campylobacter* isolates and type strains used in this study are listed in Table 1. They were cultured at 37 °C on 5% (v/v) horse blood agar plates in a Variable Atmosphere Incubator (Don Whitley Scientific) which maintained a micro-aerobic atmosphere of (by vol.) 5% O₂, 5% CO₂, 2% H₂ and 88% N₂. All strains were tested for catalase production, cytochrome oxidase production and nitrate reduction; and for selenite reduction, which differentiates *C. helveticus* from *C. upsaliensis* (Stanley *et al.*, 1992).

Nucleic acid techniques. Preparation of genomic DNA, Southern blots, and filter hybridization were done as described previously (Stanley *et al.*, 1992) except that the membrane filters were washed finally in 0.2 × SSC, 0.1% SDS at 65 °C. Genomic dot-blots were prepared by vacuum-blotting of denatured genomic DNA (5 µg) onto a Hybond-N nylon membrane filter (Amersham) using a Hybri-dot manifold (Bethesda Research Laboratories). Probe DNA was prepared from the recombinant plasmid pCH1 (Stanley *et al.*, 1992) by excising the cloned 0.8 kbp *Hind*III fragment from the vector pUC19 by standard methods (Sambrook *et al.*, 1989) and labelling with biotin-16-dUTP by random-priming.

DNA sequencing following PCR amplification of the 16S rRNA genes (1500 bp and 1650 bp respectively) of NCTC 12470^T and NCTC 12838 was carried out as previously described (Stanley *et al.*, 1992).

The PCR assay for *C. helveticus* and/or *C. upsaliensis* was made by subjecting 100 ng genomic DNA (purified as above) to PCR in a final reaction volume of 100 µl containing 10 mM Tris/HCl (pH 8.8); 200 µM dNTPs; 0.4 µM of each primer (see Results); 50 mM KCl; 1.5 mM MgCl₂; 0.1% Triton X-100 and 2.5 units of *Taq* XL DNA polymerase (Northumbria Biologicals). Samples were overlaid with 100 µl light mineral oil (Sigma) and subjected to 25 cycles of amplification on a PCR heating block (OmniGene, Hybaid) with the following cycle conditions: denaturation (94 °C, 30 s); annealing (50 °C, 30 s); extension (72 °C, 1 min). Amplicons were analysed by electrophoresis of a 10 µl aliquot through a 1% (w/v) agarose submarine gel.

A 550 bp probe for RFLP analysis at 16S rRNA gene loci was prepared as previously described for *Helicobacter pylori* (Linton *et al.*, 1992). Genomic DNA was digested with *Hind*III, for which there is no restriction site in the probe fragment.

Phylogenetic analysis of the 16S rRNA gene sequence of NCTC 12470^T. The 16S rRNA sequence for the nominated type-strain was entered into 'RNA', a program for analysis of 16S rRNA data written in Microsoft Quick BASIC for use on IBM-PC compatible computers, and aligned as previously described (Paster & Dewhirst, 1988). The reference database contained approximately 50 sequences of *Campylobacter*, *Arcobacter*, *Helicobacter* and *Wolinella* and 300 sequences of other Eubacteria. Similarity matrices were constructed from aligned sequences by using only those base positions for which 90% of the strains had data. The similarity matrices were corrected for multiple base changes (Jukes & Cantor, 1969) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The reference strains with their GenBank accession numbers were those previously described for the phylogenetic analysis of *H. canis* (Stanley *et al.*, 1993), except for *C. jejuni* subsp. *doylei* NCTC 11951^T (accession number L14630),

Campylobacter sp. 'UPTC' NCTC 11845 (L14631), '*C. mucosalis*-like' strain CCUG 20705 (L14629), *C. showae* ATCC 51146^T (L06974) and *Campylobacter* sp. PGC 40-6AT (L04318).

Secondary structure of the IVS found in NCTC 12838.

Prediction of secondary structure of lowest free energy for the IVS was made according to the Zuker & Stiegler (1981) algorithm in the DNASIS program.

RESULTS

Preliminary characterization of the strain sets

The first set comprised nine strains of *C. helveticus* including NCTC 12470^T (Stanley *et al.*, 1992). The second set (of Swiss origin) comprised ten strains which were identified by hybridization of culture-lysate dot blots (Burnens & Nicolet, 1992) with DNA of NCTC 12470^T: they included one canine (E504-90) and nine feline isolates. The third set (of UK origin) comprised three canine and nineteen feline isolates (see Table 1), received as *C. upsaliensis* from Dr P. Griffith, University of Reading, UK, and designated for the purposes of this study as '*C. upsaliensis*-like'. The third set was used to evaluate a PCR identification assay for *C. helveticus*, and its discrimination from *C. upsaliensis* (see below). These 22 isolates were catalase-negative or weakly positive ('CNW'), oxidase positive and reduced nitrate.

Nucleotide sequencing of the 16S *rrn* gene of *C. helveticus* and design of species-specific PCR primers

The DNA sequence of the gene from NCTC 12470^T was determined, following PCR amplification of a fragment (nucleotides 7 to 1510), by using eight primers corresponding to phylogenetically-conserved regions (Stanley *et al.*, 1993). The resulting sequence (GenBank accession number U03022) when compared with other eubacterial 16S rRNA sequences in our database, indicated that NCTC 12470^T was a *Campylobacter* species closely related to *C. upsaliensis*. Comparisons were made at 1378 base positions, for which over 90% of the strains had data, and corrected for multiple base changes by the method of Jukes & Cantor (1969). A phylogenetic tree obtained using the neighbour-joining method is shown in Fig. 1. There were three major clusters in the phylogenetic tree. The first cluster was composed of *Campylobacter* species and two generically misnamed *Bacteroides* species. The second cluster contained the four *Arcobacter* species. The third cluster consisted of *Helicobacter* species, '*Flexispira rappini*' and *Wolinella succinogenes*.

The nearest neighbouring species to *C. helveticus* was *C. upsaliensis* (98.2% sequence similarity) and the new species was located in the cluster of thermotolerant *Campylobacter*s, which includes *C. jejuni* subsp. *jejuni* (97.2% sequence similarity), *C. jejuni* subsp. *doylei* (97.1% sequence similarity) and *C. coli* (96.3% sequence similarity). The other *Campylobacter* species exhibited the following sequence similarities: *C. lari* (96.8% similarity); *C. fetus* subsp. *fetus* (93.1% similarity); *C. hyointestinalis* (93.5% similarity); *C. mucosalis* (92.5% similarity); *C. concisus* (92.1% similarity); *C. curvus* (91.7% similarity); *C.*

Fig. 1. Phylogenetic tree. *C. helveticus* and 33 reference species of *Campylobacter*, *Arcobacter*, *Helicobacter* and *Wolinella* were analysed by 16S rRNA sequence similarity. The scale bar represents 5% difference in nucleotide sequence as determined by measuring the lengths of the horizontal lines connecting any two species. T, type strain.

sputorum subsp. *bubulus* (91.4% similarity); *C. showae* (91.2% similarity) and *C. rectus* (91.6% similarity). The *Helicobacter* species exhibited from 87.7% similarity (*H. canis*) to 85.3% similarity (*H. fennelliae*).

The sequence was examined for unique regions which might act as priming sites for the species-specific amplification of a PCR product internal to the gene. The oligomer 5'-CCC ATA CTC CTA TTT AGC AT-3', which is found at nucleotides 176 to 196 of the *C. helveticus* and *C. upsaliensis* sequences, was predicted as a suitable forward primer. The oligomer 5'-GAT TCC ACT GTG GGG GA-3', corresponding to nucleotides 1462 to 1478 of both species, was predicted as a reverse primer for a potential PCR product of ~1260 bp from these two, but not from any other *Campylobacter* species. If it was substituted by the oligomer 5'-ATA TCT CTA TAA GGT TCT TA-3' (nucleotides 991 to 1010 of the *C. helveticus* sequence) a PCR product of somewhat over 800 bp was predicted from *C. helveticus* alone.

Rapid species-specific PCR identification of *C. helveticus*

Amplification of species-specific PCR product(s) was compared with hybridization data obtained with a recombinant DNA probe, pCH1 (Stanley *et al.*, 1992), by examining a set of '*C. upsaliensis*-like' isolates (see above).

As shown in Fig. 2(a) and Table 1, pCH1 positively identified 12/22 strains in this subset as *C. helveticus*. The remaining 10/22 strains and the *C. upsaliensis* control did not hybridize with pCH1. A parallel PCR experiment was carried out using the *C. helveticus*/*C. upsaliensis* primer pair (see above). A product of ~1260 bp was amplified from 16/22 strains, a product of ~1410 bp was amplified from three strains, and products of both sizes were amplified from two strains (Fig. 2b). These were termed CHCU amplicons (Table 1). There was a single strain (C129-92) from which no CHCU product(s) were amplified and no products were amplified (50 °C annealing temperature) from DNA of the type strains of *C. jejuni* (subsp. *jejuni* and *doylei*), *C. coli*, *C. lari*, *C. hyointestinalis*, *C. fetus* (subsp. *fetus* and *venerealis*), *C. mucosalis*, *C. concisus*, *C. sputorum* (subsp. *sputorum*, *faecalis* and *bubulus*), *C. curvus*, *C. rectus* or any *Helicobacter* species. Therefore, the CHCU reaction was judged to be specific for *C. upsaliensis*/*C. helveticus*.

On the other hand, the primer pair designed for specificity to *C. helveticus* amplified products of ~830 bp and/or ~980 bp (termed CH products in Table 1). The annealing temperature for species-specific amplification of this CH product was determined to be 52 °C – at this temperature, products were amplified from *C. helveticus* but not from any of the above-cited species of *Campylobacter* or *Helicobacter*. Those strains which had failed to reduce

Fig. 2. (a) Rapid molecular identification of *C. helveticus* by DNA hybridization. Genomic dot-blot hybridized with probe pCH1. Position A1 contained DNA of strain C121-92; position A2 of strain C123-92; A3, C126-92; A4, NCTC 12846; A5, C128-92; A6, C129-92; A7, C130-92; A8, C131-92; A9, C132-92; A10, C133-92; A11, C135-92; A12, C136-92; B1, C137-92; B2, C138-92; B3, NCTC 12849; B4, C140-92; B5, C141-92; B6, C142-92; B7, C143-92; B8, C144-92; B9, C145-92; B10, NCTC 12838; B11, *C. upsaliensis* NCTC 11541^T; B12, *C. helveticus* NCTC 12470^T. (b) PCR identification of *C. helveticus* and *C. upsaliensis*. The primer pair designed for specificity to both species amplified products of 1260 bp, 1410 bp or of both sizes. The order of strains on the 1% agarose gel is the inverse of that in (a), i.e. track 1 contained products from strain NCTC 12470^T; track 2 from NCTC 11541^T; track 3, NCTC 12838; 4, C145-92; 5, C144-92; 6, C143-92; 7, C142-92; 8, C141-92; 9, C140-92; 10, NCTC 12849; 11, C138-92; 12, C137-92; 13, C136-92; 14, C135-92; 15, C133-92; 16, C132-92; 17, C131-92; 18, C130-92; 19, C129-92; 20, C128-92; 21, NCTC 12846; 22, C126-92; 23, C123-92; 24, C121-92. (c) PCR identification of *C. helveticus*. The primer pair designed for specificity to *C. helveticus* amplified products (termed CH) of 830 bp, 960 bp or of both sizes. Order of strains as in (b).

selenite in preliminary phenotypic tests, and reacted with pCH1, produced CH amplification products. Amplicons of ~830 bp and ~980 bp occurring alone or in combination are shown in Fig. 2(c). Those strains from which the CH amplicon of ~830 bp was generated produced a CHCU amplicon of ~1260 bp. Those strains from which a CH amplicon of ~980 bp was generated produced a CHCU amplicon of ~1410 bp. Those strains from which both CH amplicons were generated produced both CHCU amplicons. Therefore 12/22 isolates were definitively identified as *C. helveticus*. All strains in the first two sets were also verified as *C. helveticus* by pCH1 hybridization and the CH amplicon assay (data not shown).

Molecular typing of *C. helveticus* by RFLP at 16S *rrn* loci

In order to estimate the copy number of the 16S rRNA genes, and with a view to outlining a potential subtyping scheme for *C. helveticus*, restriction site variation was examined at the 16S *rrn* loci. Genomic Southern blots (*Hind*III digests) were probed with the 550 bp intragenic fragment, which has no *Hind*III site. Provided that hybridizing bands are sufficiently small to exclude the

possibility that any one might carry closely-linked copies, the number of bands in a *Hind*III digest should correspond to the gene copy number. This analysis was made for the 20 strains of *C. helveticus* listed in Table 1 and for 12 *C. helveticus* strains positively identified from the '*C. upsaliensis*-like' set by CH amplicon production and positive pCH1 hybridization.

All strains exhibited homologous bands sized between 1.7 and 2.4 kbp. In 29/31 strains three bands were detected, and in 2/31 strains, two bands were observed, but comigrating fragments could not be excluded (Fig. 3, tracks 4 and 6). These results are consistent with a copy number of three in over 90% of the strains. Nine different 16S rRNA gene profiles were found among the *C. helveticus* strains (Fig. 3). They were composed of combinations of seven homologous *Hind*III fragments, six of which (1.7, 1.85, 2.0, 2.1, 2.2 and 2.4 kbp) were present in more than one group of strains. The nine profiles occurred with varying frequency: one major and a number of minor subtypes were identified. The commonest profile (Fig. 3, track 10) was found in 18/31 strains, the next most prevalent profiles (Fig. 3, tracks 9 and 8) in 3/31 strains and the rest in single strains only.

Fig. 3. 16S *rrn* gene type profiles of *C. helveticus*. Genomic Southern blot made with *Hind*III-digested DNA hybridized with a 550 bp internal fragment of the 16S *rrn* gene. Track 1 contained DNA of strain NCTC 12849; track 2, NCTC 12848; tracks 3 and 9, NCTC 12845; track 4, NCTC 12471; track 5, NCTC 12847; track 6, NCTC 12846; track 7, NCTC 12472; track 8, NCTC 12838. Track 10 contained DNA of NCTC 12470^T, exhibiting the most prevalent profile, termed RT I.

Characterization of an IVS in the 16S rRNA gene of five strains

Three of the isolates identified as *C. helveticus* in Fig. 2(c) produced an enlarged CH amplicon of ~980 bp as opposed to the amplicon of ~830 bp found in NCTC

12470^T (Fig. 2c, tracks 3, 5, 9 vs track 1). Two of the isolates produced CH amplicons of both sizes (Fig. 2c, tracks 10 and 21). The enlarged 16S rRNA gene of strain NCTC 12838 (Fig. 2c, track 3) was subjected to sequence analysis (see Methods) and shown to differ from that of NCTC 12470^T due to the presence of inserted DNA following nucleotide 210. The sequence following this anomalous 148 bp insert was identical to that found after nucleotide 212 of the NCTC 12470^T sequence (data not shown). There was a single base pair deletion at position 211 of the conventional gene sequence. The 148 bp intervening sequence (GenBank accession number U03021) contained no ORF, and when analysed for RNA secondary structure could be folded into a configuration with one major (nucleotides 4/22 and 127/145) and three minor stem-loops (Fig. 4). Examination of this sequence and its flanking regions revealed no signals for intron processing, and the insert was therefore designated an 'intervening sequence' or IVS.

In order to ascertain whether the enlargement of the gene in the other four strains was due to an IVS of similar size and location, primers corresponding to nucleotides 101–120 (IVSF1) and 241–256 (IVSR2) of the 16S rRNA gene were employed for PCR analysis. Whilst an amplicon of ~155 bp was generated from NCTC 12470^T as predicted (Fig. 5a, track 1), amplicons of ~305 bp were generated from NCTC 12838, C144-92 and C140-92 (Fig. 5a, tracks 2–4). Amplicons of both sizes were generated from the two strains previously predicted to have both classes of

Fig. 4. Secondary structure of the 148 bp IVS found in the 16S rRNA gene of *C. helveticus* NCTC 12838. The RNA secondary structure, derived as described in Methods, was located at about nucleotide 210 of the gene, using the *E. coli* numbering system (Brosius *et al.*, 1978). Solid lines denote hydrogen bonds, whilst single G–U hydrogen bonds are represented by ●. The sugar-phosphate backbone is denoted by broken lines, and has been extended (for visual clarity) between nucleotides 106/107 and 121/122.

Fig. 5. PCR analysis of enlarged genes present in five strains of *C. helveticus*. (a) Agarose gel (1.5%) analysis of PCR amplicons generated with primer pair IVSF1 and IVSR2 from NCTC 12470^T (track 1), NCTC 12838 (track 2), C144-92 (track 3), C140-92 (track 4), NCTC 12849 (track 5) and NCTC 12846 (track 6). *Bst*EII-generated fragments of phage λ are shown as size markers in track 7. (b) Diagrammatic representation of the results. Scale marks represent 100 bp intervals.

gene (Fig. 5a, tracks 5 and 6 vs Fig. 2c, tracks 10 and 21). These primers and amplicons are represented in Fig. 5(b). It was concluded that the five strains of *C. helveticus* concerned contain a 16S rRNA gene enlarged by the presence of an IVS of similar size, located in the same region.

DISCUSSION

The 16S *rrn* gene of *C. helveticus* NCTC 12470^T exhibited 1.8% nucleotide sequence difference from that of *C. upsaliensis* and 2.8–2.9% sequence difference from that of *C. jejuni*. The phylogenetic position so obtained (particularly the close relationship to *C. upsaliensis*, and the general placement among the thermotolerant species) agrees with previous results from the numerical analysis of cellular proteins, and from total DNA hybridization (Stanley *et al.*, 1992). Therefore it is interesting to compare the biology of *C. helveticus* with that of *C. upsaliensis*. Both these *Campylobacter* species were first found in domestic

pet animals: *C. upsaliensis* in dogs (Sanstedt *et al.*, 1983) and cats (Fox *et al.*, 1989) and *C. helveticus* in cats (Stanley *et al.*, 1992) and rarely in dogs (this report). *C. upsaliensis* was subsequently shown to be a significant agent of human disease (Lastovica *et al.*, 1989; Patton *et al.*, 1989; Taylor *et al.*, 1989; Goossens *et al.*, 1990). The possible role of *C. helveticus* in human disease should therefore be examined, since it is sensitive to some selective media and conditions, and may also be easily misidentified as *C. upsaliensis*. For example, 45% of the third set of isolates of veterinary origin designated '*C. upsaliensis*-like' by bacteriological criteria were shown by PCR and DNA probe identification to be *C. helveticus* strains. Isolates identified bacteriologically as *C. upsaliensis* should therefore be examined by the CH amplicon test.

We suggest that such methods are more definitive than conventional phenotypic tests for these biochemically inert microaerophilic *Campylobacter*s. The availability of two species-specific PCR tests permits identification of *C. helveticus*–*C. upsaliensis* on the one hand, and differentiation of the two species on the other. Another advantage of PCR in this case is that positive identification is rapidly obtained from very little biological material. Clinical samples might be investigated in this way, removing the need for primary bacteriological isolation and cultivation, which is often difficult and time consuming for these species. Such rapid screening of clinical material could elucidate the carriage and disease association of *C. helveticus* in man. Although the 16S rRNA sequence has previously been used for the design of species-specific oligonucleotide probes (Wesley *et al.*, 1991), and primers for amplification of a 426 bp PCR product from *C. jejuni*, *C. coli* and *C. lari* have also been described (Giesendorf *et al.*, 1992), the primers described here yield the first specific PCR amplicons described for a single *Campylobacter* species.

Diverse probes have been employed to characterize polymorphism (RFLPs) at rRNA loci. They have included rRNA itself (Grimont & Grimont, 1986), the cloned *E. coli* (5S–16S–23S) *rrnB* operon (Altwegg *et al.*, 1989; Brosius *et al.*, 1978) or an intragenic PCR-generated fragment of the 16S *rrn* gene alone (Linton *et al.*, 1992). Better-defined probes permit conceptually simpler and accountable 'ribotyping' schemes to be developed. Results obtained here with a 550 bp internal fragment of the 16S rRNA gene are both simple and discriminatory. They indicated that the copy number of the gene in *C. helveticus* was generally three, in agreement with results previously obtained for *C. jejuni* and *C. coli* (Kim *et al.*, 1992; Taylor *et al.*, 1992). The nine profiles based on variation of *Hind*III sites in and around the 16S rRNA gene of *C. helveticus* form the basis of a molecular typing scheme for the species, an approach which is also applicable to major agents of human disease such as *C. jejuni*. There was no association between the geographical origin of the isolates (Switzerland vs UK) and 16S rRNA gene profile. The most prevalent profile (*Cb*-RI) and a number of rarer or unique profiles were found among strains from either country.

The principal finding to emerge from this study is that

certain strains of *C. helveticus* contain an IVS of ~ 150 bp in the 16S rRNA gene. This is a first report of the presence of inserted DNA in the phylogenetically important 16S rRNA gene of a eubacterium. Only one exception to the uniform size of this gene has been described so far. This is in the archaeon *Pyrobaculum aerophilum* (Burggraf *et al.*, 1993), where an intron of 713 bp has been found in the gene. Intervening sequences (IVSs) of unknown function have however been demonstrated in the 23S rRNA gene, in the eubacteria *Salmonella typhimurium*, *S. arizonae* and *Yersinia enterocolitica* (Burgin *et al.*, 1990; Skurnik & Toivanen, 1991). The stem-loop structure of the *C. helveticus* IVS bears comparison with those found in the 23S rRNA gene of *S. typhimurium* and *S. arizonae* (Burgin *et al.*, 1990), particularly with respect to the sequence and structure of the principal stem-loop. This region was shown to be the site of *in vivo* cleavage by RNaseIII, a processing enzyme which is thought to recognize secondary structural features of duplex stems rather than simple nucleotide sequences. This would be consistent with excision of the 148 bp IVS during maturation of 16S rRNA in these *C. helveticus* strains. As to the occurrence of strains (genes) of *C. helveticus* with and without the IVS, the question arises whether the enlarged 16S rRNA gene represents an ancestral state, or whether it is 'recently' acquired in some strains (genes) by recombination or rearrangement. These questions can only be resolved by further studies of *C. helveticus* and related species containing similar 16S rRNA gene insertions.

At the time of writing, similarly enlarged genes have been detected in certain other eubacterial species belonging to the genera *Campylobacter* and *Helicobacter*. The sequences, structures and significance of these insertions will be reported elsewhere.

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NOTE ADDED IN PROOF

While this paper was in press, a paper by Van Camp *et al.* (*Syst Appl Microbiol* 16, 361–368, 1993) was published, in which an insert in the 16S rRNA gene of *Campylobacter sputorum* was reported.

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