Differentiation of Campylobacter coli and C. jejuni by length and DNA sequence of the 16S–23S rRNA internal spacer region

Henrik Christensen, Kirsten Jørgensen and John Elmerdahl Olsen

The internal spacer region (ISR) between the 16S and 23S rRNA genes of Campylobacter was investigated by PCR fragment length typing and DNA sequencing of clinical and chicken wild-type isolates. PCR fragment length typing showed one fragment of 859 nt in length for the 12 strains of Campylobacter coli investigated. Thirty-six of the Campylobacter jejuni subsp. jejuni strains possessed one fragment, which varied in size between 727 and 802 nt. Three strains showed two fragments between 501 and 923 nt. Strains of C. jejuni subsp. doylei, Campylobacter lari and Campylobacter upsaliensis possessed one or two fragments with lengths different from those of C. coli and C. jejuni subsp. jejuni. DNA sequences were obtained from 54 nt downstream of rrs up to rrl of four strains of C. coli, eight strains of C. jejuni subsp. jejuni, and one strain each of C. jejuni subsp. doylei and C. lari, selected to represent the different biotypes of Campylobacter. ISR lengths determined by PCR fragment length typing and DNA sequencing corresponded for 12 strains. For two strains of C. coli, PCR fragment length typing underestimated ISR lengths by 159 and 193 nt, probably related to incomplete resolution of the distal helical structures, which were not fully denatured during PAGE. For the 14 strains and the published C. jejuni subsp. jejuni sequence, the first 206–211 nt were conserved and included the two tRNA genes in the characteristic tRNAGlu to tRNAIle order separated by a short 8–9 nt spacer region. Within the region downstream of tRNAIle, conserved regions were identified which allowed a separation of C. lari from C. coli and C. jejuni but not separation of C. coli from C. jejuni. The 69–282 nt longer variable regions in C. coli strains allowed separation of this species from C. jejuni, confirming results obtained by PCR typing. Certain nucleic acid positions in variable regions were related to the Lior biotypes. Sequence information from ISRs of more strains is needed to ascertain if separation of species and biotypes will be possible for diagnostic purposes.

Keywords: Campylobacter coli, Campylobacter jejuni, rRNA, internal spacer region, ISR

INTRODUCTION

The identification of Campylobacter coli and Campylobacter jejuni is limited by low discrimination of phenotypic tests (On, 1996). Genotyping has the potential to improve separation, including typing below the species level (Owen et al., 1990; Steele et al., 1998); however further evaluation and standardization of genotyping methods are needed (Wassenaar & On, 1998). It is possible to standardize PCR-based methods for identification and typing but improvements are needed in relation to specificity (On, 1996). Direct sequencing of DNA for identification and typing of campylobacters is
another alternative that might become favourable with the increasing automatization of this technique.

The internal spacer region (ISR) between 16S rRNA and 23S rRNA genes has been used as a target for PCR-based identification and typing of many bacteria (Gürtler & Stanisich, 1996). In *Escherichia coli* the sequences which form the strong base-paired stems at each end of the mature rRNA genes are conserved ( Pace & Burgin, 1990; Srivistava & Schlessinger, 1990). Other regions of 16S–23S rRNA gene spaces are highly variable and have been used for genotyping of bacteria by fragment length profiling, restriction fragment length profiling or DNA sequencing (Gürtler, 1993; Gürtler & Stanisich, 1996; LeBlond-Bourget et al., 1996; Zavaleta et al., 1996).

*C. jejuni* possesses three copies of *rrn* genes (Kim et al., 1993). Within the *rrnA* operon, rRNA genes are separated by a 805 bp long spacer region which includes the *rrn* genes (Kim et al., 1993, 1995). Not all ribosomal genes may be contained in all *rrn* operons. In *C. jejuni* strain NCTC 11168, *rrs* (16S rRNA) genes were separated from *rrl* (23S rRNA) genes and only one operon remained intact with linkage between genes (Taylor et al., 1992; Newnham et al., 1996).

The aim of the present investigation was to analyse ISRs at the fine structural level to obtain basal knowledge of the genetic diversity of wild-type and reference strains representing the phenotypic spectrum of *C. coli* and *C. jejuni*, and to evaluate this information for differentiating the two species.

**METHODS**

**Bacterial strains and media.** *C. jejuni* subsp. *jejuni* (*n* = 39), *C. jejuni* subsp. *doyleri* (*n* = 2) and *C. coli* (*n* = 12) were selected to cover six biotypes and 28 serotypes. Two strains of the related *C. lari* and one strain of *C. upsaliensis* were included for comparison (Table 1, Fig. 2). Bacteria were cultured from the freeze-dried state on Blood Agar (Base no. 2, Oxoid) in a micro-aerophilic atmosphere of 85% H₂/10% CO₂/5% O₂ for 2 d at 37°C.

**DNA extraction.** Colonies were scraped from plates and resuspended in 0.5 ml TE buffer (50 mM Tris, 50 mM EDTA, pH 8). Cells were washed in TE buffer by centrifugation and lysis was initiated by addition of 50 µl of a solution containing 10 mg lysozyme ml⁻¹. After incubation at 37°C for 30 min, 50 µl of a solution containing 10 mg Proteinase K ml⁻¹ in TE buffer and 20 µl 10% SDS pH 7.2 was added, gently mixed and further incubated for 2 h at 56°C. Cell debris and protein were precipitated with 297 µl 3 M potassium acetate. After centrifugation at 16,000 g for 10 min, the supernatant was added to 0.54 vol. 2-propanol, gently mixed and centrifuged again at 16,000 g for 10 min. The pellet was washed twice in ice-cold ethanol by centrifugation, vacuum-dried and resuspended in sterile distilled and filtered ultrapure water.

**PCR amplification.** Three nested sets of oligonucleotide primers were used to amplify the spacer between 16S and 23S rRNA genes. The criterion for the identification of fragments was the presence of the same number of fragments with all three PCR primer-sets in order to eliminate PCR products generated by misannealing of primers. Oligonucleotide primers were designed with identity to the *C. jejuni rrnA* sequence (Kim et al., 1995). The primer for forward directional amplification was 16S-1498f (5’ GGTGTGAGTACCTCCTTT) in all three sets and was located on the 16S gene. The three reverse sets of primers, located on the 23S rRNA gene, were 23S-30r (5’ CAGGGCATCCACCGT), 23S-123r (5’ GGTTG-CGCCCATTCGG) and 23S-494r (5’ CTTTTGCGCTTCTTTCCTTCTC) (Fig. 1). Fragments generated with primer-sets 1, 2 and 3 have overlaps with 16S and 23S gene sequences of 45, 138 and 504 nt, respectively. The primer sequences were searched for similarity to *Campylobacter* DNA sequences deposited in the GenBank/EMBL database. At least five mismatches to genes other than *rrn* were found. The PCR primers therefore were predicted to amplify *rrn* regions only. For analysis on the ALF DNA sequencer (Pharmacia Biotech), one of the primers in each primer-set was labelled with FITC. PCR amplification was performed as described by Vogel et al. (1997) with the modifications of 0.1 µM concentration of each primer and 1 min annealing time.

**Analysis of fragments.** Fragments amplified by PCR were separated by denaturing PAGE on the ALF DNA sequencer. The size-marker Sizer 50-500 (Pharmacia Biotech) and a size-marker with 367, 583, 641, 740, 905 and 1161 nt double-stranded DNA were included on the gel. The size-marker of 367–1161 nt long fragments was made by PCR amplification of 23S rRNA gene from *Salmonella bongori* strain BR1839 by the primer-sets 114f (5’ CCGAATGGGGAAACCC)/484r (5’ GAGGGAAAGGCGAAG), 1939f (5’ GTAGGCGATT-CCTTGTC)/2498r (5’ CCTGATGTCGGCT), 481f (5’ GAGGGAAAGGCGAAG)/1104r (5’ AGAAGGCTAATTGAGT), 1939f/2654r (5’ AGTACGGAGGACCG), 1608f (5’ AACCGACACAGGTGG)/2498r, and 481f/1608r (5’ AACCGACACAGGTGG), respectively, with both primers labelled by FITC. The lengths of fragments were calculated by comparison with the published 23S rRNA gene sequence with GenBank accession number U77927.

Polyacrylamide gels (ReadyMix gel, Pharmacia Biotech) composed of 6% acrylamide, 7.0 M urea and 100 mM Tris/borate (pH 8.3) were cast with 0.5 mm spacers and loaded with PCR product which had been mixed with an equal volume of Stop-mix (AutoRead sequencing kit, Pharmacia Biotech), denatured at 94°C for 2 min, spun down in a microcentrifuge and immediately cooled on ice. Electrophoresis was performed at 1500 V and 45°C for 700 min. Peak positions and intensity were analysed by the program Fragment Manager (Pharmacia Biotech). Fragment lengths were reduced to allow for the overlaps with the 16S and 23S rRNA genes created during PCR amplification.

**Partial sequencing of spacer fragments.** PCR-amplified fragments were purified on Microspin columns (Pharmacia Biotech) and cycle-sequenced (Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing kit, Amersham) on an ALF sequencer (Pharmacia Biotech) using the primers 16S-1498f, 23S-123r (see above) and 289f (5’ CTTTAAAATCTAAAGCCTT). Fragments were purified with the published sequence for the *rrnA* operon of *C. jejuni* strain TGH 9011 (GenBank accession no. Z29326). Alignment and analysis of sequence structure were performed by use of the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, USA) including the use of Mfold (Zuker, 1989) and GeneCompar (Applied Maths, Kortrijk, Belgium).
Internal spacer region of Campylobacter

**rrnA operon region of C. jejuni TGH 9011**

![Diagram of rrnA operon region]

**Alignment of ISR DNA sequences**

![Alignment of ISR DNA sequences]

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**RESULTS AND DISCUSSION**

**Typing by PCR fragment length profiling of ISRs**

PCR amplification of ISRs allowed separation of *C. coli* from *C. jejuni* subsp. *jejuni* by the length and number of fragments (Fig. 2). The 12 strains of *C. coli* showed one ISR with mean length of 859 nt. Thirty-six out of the 39 *C. jejuni* subsp. *jejuni* strains showed only one fragment, but this varied in size between 727 and 806 nt. The remaining three *C. jejuni* subsp. *jejuni* strains showed two fragments of which the shorter was between 501 and 735 nt and the longer between 763 and 923 nt,

![Table of ISR fragment lengths]

**Fig. 2.** Typing of Campylobacter based on PCR amplification of ISRs. The cross hatching represents fragment length variation between strains of each species. ND, Not determined.
clearly separated from the length of the one C. coli fragment. One C. jejuni subsp. doylei strain showed one fragment of 828 nt, whilst the other strain showed two fragments of 794 and 986 nt. C. lari possessed one fragment of 678 nt or two fragments of 678 and 831 nt. C. upsaliensis showed two fragments with lengths of 633 and 653 nt (only one strain).

The number of fragments was confirmed by use of the three nested primer-sets. The data reported in Fig. 2 are means of fragment lengths obtained with primer-sets 1 and 2 because fragment lengths obtained with these primer-sets were comparable. Up to 91 nt underestimation was obtained by use of primer-set 3 as the longest fragments were greater than the length of the 1161 nt size marker.

**Correspondence between PCR fragment length and DNA sequence analysis of ISRs**

Selected strains from the PCR fragment length profiling of ISR were further investigated by sequencing (Table 1). DNA sequences of four C. coli, nine C. jejuni and one C. lari strain were obtained. These strains possessed only one spacer fragment (Table 1). Sequences were determined from position 1830 of the C. jejuni TGH 9011 rrnA sequence (GenBank accession no. Z29326), 54 nt downstream of rrS up to the rrl gene (see Fig. 1). The lengths of ISRs determined by PCR typing and measured as DNA sequence corresponded within the experimental precision of 10 nt except for C. coli strains JEO 2772 and JEO 2777. Low accuracy was also found with C. jejuni subsp. jejuni strain NCTC 11392, where the spacer length was overestimated by 60 nt by PCR typing compared to sequencing. When C. coli strains JEO 2772 and JEO 2777 were sequenced, the lengths were determined as 999 and 967 nt, respectively (Table 1). The underestimation found by PCR typing might be related to incomplete resolution of the distal helical structures which were not fully denatured during PAGE. The mobility of such structures is known to be faster than that of single-stranded DNA and thus they will be underestimated in length (Sambrook et al., 1989).

Sequences of C. coli and C. jejuni were conserved in the first 51 nt upstream from the trNA_Ala gene, through the trNA_Ala and trNA_iLe genes including the 8–9 nt long trNA spacer (data not shown). Base number 42 (G) of trNA_iLe in the C. jejuni TGH 9011 rrnA sequence was absent from all of the campylobacter strains sequenced in the present study.

Downstream from trNA_iLe four conserved regions of 15, 120, 40 and 84 nt were identified (Fig. 1). Within these regions and the conserved region upstream of trNA_Ala variation between the nine strains of C. jejuni subsp. jejuni was 0–1.3%. Variation in the same range was found between C. coli strain JEO 2772 and C. jejuni subsp. jejuni strains and between strains CIP 70.80 and NCTC 11333 of C. coli. The variation was 1.6–2.9% between C. jejuni subsp. doylei strain NCTC 11951 and the C. coli strains and 2.9–3.6% between strain NCTC 11951 and the C. jejuni subsp. jejuni strains. Variations of 0.3–3.6% were found between C. coli strains and up to 4.6% variation between C. coli and C. jejuni subsp. jejuni. The variation between C. lari and C. coli and C. jejuni was 11.5–13.8%. The sequence variation between conserved parts of ISRs was more than twice the variation of 1.9–2.2% found between 16S rRNA sequences of C. coli and C. jejuni (Paster & DeWhirst, 1988; Thompson et al., 1988). The variation of up to 14% between C. lari, C. coli and C. jejuni was much higher than the 22–37% variation determined by 16S rRNA sequence comparison (Paster & DeWhirst, 1988; Thompson et al., 1988). The variability between C. coli, C. jejuni and C. lari within conserved regions of ISRs (trNA genes excluded) therefore was more than twice that of 16S rRNA sequences.

One of the most likely reasons for conservation of parts of ISRs is the preservation of processing stems for rRNAs. Based on the published sequence of C. jejuni strain TGH 9011, a processing stem could form between the region between rrs and trNA_Ala genes with the first 110 bases downstream of trNA_iLe (79% complementarity), or between the latter region and the region upstream of rrs (80% complementarity) (see Fig. 1). Complementarities of 75–83% were determined in all strains for the first possibility.

Divergence in sequence length of variable regions was responsible for the variable ISR lengths observed by PCR typing. These sequence mosaics crossed the species border of C. coli and C. jejuni as the first 25 nt insert downstream from trNA_iLe was found in CIP 70.80, NCTC 11333 and NCTC 11392 and the region from nt 75 to 210 (consensus numbering according to Fig. 1) was deleted in strains NCTC 11333 and NCTC 11392. The region from nt 566 to 755 was only found in C. coli strains, explaining the longer fragment length identified by PCR typing. For strains with only one ISR fragment, C. coli was separated from C. jejuni on the basis of the 152–193 nt insertion upstream of rrl.

**Signature nucleic acid positions**

On the basis of variable regions it was possible to identify 19 signature nucleic acid positions where strains of C. jejuni subsp. jejuni could be separated according to the four Lior biotypes (Lior, 1984) (Fig. 3). For the first 11 positions up to consensus position 303, variations occurred at random between biotypes. However, for the last eight signatures biotype I differed from II, III and IV.

The correlation between DNA sequence and biotype is in accordance with the high correlations observed between PFGE groups and biotypes (Steele et al., 1998) and between ribotyping, biotyping and serotyping of C. jejuni and C. coli (Owen et al., 1990), which point to a clonal population structure. The clonal population structure of C. coli and C. jejuni has not been investigated in detail; however 88% of C. coli strains were in one sero-, ribo- and flagellin-type specific clone (Stanley et al., 1995), and 62% of Danish C. jejuni
isolates were distributed in three serotypes (Nielsen et al., 1997). If the mosaic pattern of DNA sequences found in ISRs had been the result of recent recombination events between *Campylobacter* species, the observed correlations with biotypes of *C. coli* and *C. jejuni* would have been very unlikely.

### Structure of *rrn* operons

Fifty out of the 56 strains analysed contained only one PCR-amplified spacer fragment. These strains were assumed to contain only one intact *rrn* operon because this was found for strain NCTC 11168 of *C. jejuni* by chromosome mapping (Taylor et al., 1992; Newnham et al., 1996) and DNA sequences determined for *C. coli* and *C. jejuni* strains were structurally similar. The presence of two or three operons with equal spacer lengths cannot be excluded. However variations would have been expected in the light of the high degree of chromosomal instability observed with *Campylobacter* (On, 1997; Wassenaar et al., 1998). Recently, all three paired copies of *rrs* and *rrl* genes were mapped pairwise within the same restriction fragments of strain NCTC 11168 (Karlsheva et al., 1998). The present study shows that the distance between two of the pairs of *rrs* and *rrl* were above PCR-amplifiable lengths. It remains to be determined which part of the ISRs there are accompanying *rrs* and *rrl* genes of split-up operons.

The ISR sequence was characterized by a low GC content of 9–38 mol% for variable regions and 22–31 mol% for conserved regions, in sharp contrast to *E. coli* where a value of 57–61 mol% was found for both these regions (Brosius et al., 1981). Such low GC contents

### Table 1. Origin, and bio- and serotype characteristics of strains analysed, and nucleotide lengths of 16S–23S rRNA ISRs obtained by PCR typing and DNA sequencing

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Origin</th>
<th>Lior biotype</th>
<th>Serotype†</th>
<th>PCR typing</th>
<th>DNA sequence‡</th>
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<td></td>
<td></td>
<td></td>
<td>HS</td>
<td>HL</td>
<td>Fragment length</td>
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<tr>
<td>JEO 2677 (3-1 a)</td>
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<td>I</td>
<td>ND</td>
<td>2</td>
<td>796</td>
</tr>
<tr>
<td>NCTC 11168 (KJ6, JEO 4128)</td>
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<td>I</td>
<td>2</td>
<td>4</td>
<td>781</td>
</tr>
<tr>
<td>JEO 2693 (12583/87)</td>
<td>Human faeces</td>
<td>I</td>
<td>ND</td>
<td>9</td>
<td>801</td>
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<tr>
<td>JEO 2762 (22-5 b)</td>
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<td>793</td>
</tr>
<tr>
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<td>6</td>
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<td>777</td>
</tr>
<tr>
<td>KJ</td>
<td>Human</td>
<td>III</td>
<td>ND</td>
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<td>780</td>
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<td>ND</td>
<td>40</td>
<td>796</td>
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<tr>
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<td>Chicken</td>
<td>IV</td>
<td>ND</td>
<td>19</td>
<td>799</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>CCUG 29406 (JEO 4161)</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
<td>678</td>
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</tr>
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</table>

* The strain designation given for the culture collection from which the strain was obtained. Alternative strain designations are given in parentheses. CCUG, Culture Collection of the University of Göteborg, Sweden; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK; JEO and KJ, Royal Veterinary and Agricultural University, Copenhagen, Denmark.
† HS, heat-sensitive (Penner); HL, heat-labile (Lior) serotypes. ND, Not determined.
‡ DNA sequence 54 nt downstream of rRNAaa from position 1830 to 2581 of *C. jejuni* *rrnA* gene (GenBank accession no. Z29326). Sequence lengths were increased by 53 nt for comparison with the full length of the 16S–23S rRNA spacer region.
provide low possibilities for stable helix formation (Srivastava & Schlessinger, 1990). The less stable AT-rich secondary structures would be important during transcription of rRNA genes (Richardson & Greenblatt, 1996) and the GC content of ISRs is at the same level as for the whole C. coli and C. jejuni genome of 30–33% (Van Damme & Goossens, 1992). The predicted helices mostly ended in loops of five nucleotides, which is in contrast to 16S and 23S rRNA where tetraloops dominate (Gutell et al., 1994).

Secondary helical formations were predicted in the regions 76–134 and 151–190 of the consensus sequence for all strains except CIP 70.80, NCTC 11353 and NCTC 11392, where this region was deleted (Fig. 1). In these strains a helix was predicted instead from nt 52 to 232 of the consensus sequence (68 nt). Helix formation was further predicted in the conserved regions from nt 277 to 312 and from nt 367 to 388. The last helix predicted in all strains was from nt 439 to 478. In C. coli, additional helices were predicted in the inserted region from positions 567 to 619 of JEO 2777, CIP 70.80 and NCTC 11353 and from 642 to 658 of strain JEO 2772 (Fig. 1). The GC content of 38 mol% for these regions was somewhat higher than the value of 12–28 mol% of the other helices. It was characteristic that the predicted helices were located within variable regions, indicating that the conserved regions without these structures could be involved in formation of processing stems for rRNAs.

A FASTA search showed low homology to the spacer regions of other bacteria. Only a sequence (GenBank accession no. 123675) listed from an unknown organism was 91–99% similar to the spacer of C. coli and C. jejuni. The paired order of tRNAa,T and tRNAe,T with the 8–9 nt short spacer and the ISR region between tRNAa,T and rrs are therefore characteristic of C. coli, C. jejuni and C. lari.

**Conclusion**

The insertion of between 152 and 193 nt found in C. coli but not in C. jejuni could provide a target for rapid separation of the two species based on PCR or DNA hybridization. The study showed diverging DNA sequences between all the strains; however investigation of more strains is needed to ascertain if certain biotypes can be recognized based on ISR signature nucleic acid positions. The association between other variable regions and well-recognized genotypic and phenotypic characters is poorly understood. For example, there were deletions within similar regions of the reference strains NCTC 11353 and NCTC 11392, representing both C. coli and C. jejuni, but not in the other predominant wild-type clinical and chicken isolates.
These deletions might represent recent genomic re-arrangements related to frequent laboratory cultivation as reported by On (1997) and Wassenaar et al. (1998).

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REFERENCES


