Occurrence and characterisation of intervening sequences (IVSs) within 16S rRNA genes from two atypical *Campylobacter* species, *C. sputorum* and *C. curvus*

A. TAZUMI*, S. NAKANISHI*, S. MEGURO*, Y. KAKINUMA*, J. E. MOORE†, B. C. MILLAR† and M. MATSUDA*

Laboratory of Molecular Biology, School of Environmental Health Sciences, Azabu University, Sagamihara, Japan; and †Department of Bacteriology, Northern Ireland Public Health Laboratory, Belfast City Hospital, Belfast, Northern Ireland, UK

Accepted: 9 February 2010

ABSTRACT

A polymerase chain reaction (PCR) method was carried out on 21 isolates of atypical *Campylobacter sputorum* (n=14) and *C. curvus* (n=7) using a primer pair to amplify the helix 11 region within 16S ribosomal RNA (rRNA) gene sequences. Following sequencing and alignment analysis, 14 *C. sputorum* (100%) and six *C. curvus* (86%) isolates were shown to carry intervening sequences (IVSs) in this region. Interestingly, the nucleotide sequences of all the IVSs were identical among the 14 *C. sputorum* isolates (n=5 *C. sputorum* biovar [bv] paraureolyticus; n=5 bv fecalis; n=4 bv sputorum). In addition, two different nucleotide lengths and sequences of IVSs were identified among the six *C. curvus* isolates. On the first prediction of the secondary structure model of the IVSs in 16S rRNA genes, stem and loop structures were identified. In the purified RNA fractions from the 20 *Campylobacter* isolates carrying IVSs, no 16S rRNA was evident. Instead, other smaller RNA fragments were identified. Thus, the primary 16S rRNA transcripts may have been fragmented in the 20 isolates. This is the first demonstration of atypical *C. sputorum* and *C. curvus* isolates carrying IVSs in the helix 11 region in 16S rRNA genes.

KEY WORDS: Campylobacter sputorum.
Campylobacter curvus.
Introns.
Genes, rRNA.

Introduction

Thermophilic *Campylobacter* species, primarily *C. jejuni, C. coli* and *C. fetus*, are Gram-negative bacteria that are the major and typical campylobacters of medical, public health or veterinary interest worldwide. Although some other minor and atypical *Campylobacter* species, such as *C. lari, C. upsaliensis* and *C. hyointestinalis*, have been associated with various infectious diseases,[1–3] even less information is available on campylobacters such as *C. sputorum* and *C. curvus*.

The amended report of *C. sputorum* and revision of its infrasubspecific (biovar [bv]) divisions has been described, including *C. sputorum* bv paraureolyticus, a urease-producing variant.[4] In addition, *C. curvus* was discovered in 1984 and classified originally as *Wolinella curva*.[5] In 1991, this organism was reclassified as *C. curvus*.[6] Recently, *C. curvus* has been found in a wide variety of clinical cases.[7,8]

All three biovars of *C. sputorum* (bubulus, fecalis and sputorum) have been shown to carry longer 16S rRNA genes (an insertion of approximately 250 base pairs [bp] in the helix 11 region).[10,11] This insertion was not present at the rRNA level and the 16S rRNA molecules were fragmented,[9] as seen in the 23 rRNA molecules.[10,11] In addition, enlarged 16S rRNA genes that contain atypical intervening sequences (IVSs) have been found in five out of 12 *C. helveticus* isolates,[12] in three *C. rectus*, two *C. curvus* and two *C. sputorum* isolates.[13] Moreover, distribution of IVSs in the 16S rRNA genes from two *C. hyointestinalis* subsp. *lawsonii* strains has also been described.[14]

In the 16S rRNA genes of *C. sputorum* and *C. curvus*, IVSs were identified in the five *C. sputorum* (bv bubulus LMG6447 [TCC33562)], bv fecalis LMG6617 [CCUG12015] and bv sputorum LMG7795^T [ATCC35980];[9] bv bubulus FDC616 and bv fecalis SUA3112)[13] and in the two *C. curvus* (ATCC35224 and SUC10)[13] isolates.

This study aims to clarify whether or not these two *Campylobacter* species contain IVSs as frequently as isolates of the other species, and whether or not the 16S rRNA molecules whose genes contain IVSs are fragmented.

Materials and methods

Twenty-one isolates comprising *C. sputorum* (n=14) and *C. curvus* (n=7) were used in the present study (Table 1). Genomic DNA was prepared from the *Campylobacter* cells by sodium dodecyl sulphate (SDS) and proteinase K treatment, phenol-chloroform extraction and ethanol precipitation.[15]

Correspondence to: Professor Motoo Matsuda
Laboratory of Molecular Biology, School of Environmental Health Sciences
Azabu University, Fuchinobe 1-17-71, Sagamihara 229-8501, Japan
Email: matsuda@azabu-u.ac.jp

Table 1. Isolates of *C. sputorum* and *C. curvus* analysed in the present study and summary on identification of the IVSs within 16S rRNA genes.

Isolate	IVS
C. sputorum bv. sputorum LMG7975	+
C. sputorum bv. sputorum LMG8535	+
C. sputorum bv. sputorum LMG10388	+
C. sputorum bv. sputorum LMG11765	+
C. sputorum bv. fecalis LMG8531	+
C. sputorum bv. fecalis LMG8532	+
C. sputorum bv. fecalis LMG8534	+
C. sputorum bv. fecalis LMG11761	+
C. sputorum bv. paraureolyticus LMG17589	+
C. sputorum bv. paraureolyticus LMG17590	+
C. sputorum bv. paraureolyticus LMG17591	+
C. sputorum bv. paraureolyticus LMG17592	+
C. sputorum bv. paraureolyticus LMG17593	+
C. curvus LMG7609	+
C. curvus LMG11033	+
C. curvus LMG11034	+
C. curvus LMG11127	+
C. curvus LMG11247	+
C. curvus LMG11249	+
C. curvus LMG13935	–

bv: biovar; IVS: intervening sequence

A PCR primer pair of fD1 (5′-GAGTTTGATCCTGGCTCAG-3′)[16] and r-*Ca*16h11 (5′-TGGACCGTGTCTCAGTTCC-3′)[17] was employed to amplify the helix 11 region in 16S rRNA gene sequences from the *Campylobacter* isolates. The PCR products, which were separated by 1% (w/v) agarose gel electrophoresis in 0.5× TBE, were purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan). The amplicons were subjected to cycle sequencing with BigDye Terminator (Applied Biosystems, Tokyo, Japan) and with the PCR primers.

Sequence analysis was performed by using the Genetyx-Windows computer software (version 9; Genetyx Co. Tokyo, Japan). Nucleotide sequences of the helix 11 region in 16S rRNA gene sequences from the 21 isolates of *C. sputorum* and *C. curvus* were compared and with the accessible sequence data from some other *Campylobacter* organisms using CLUSTAL W software (1.7 program),[18] which was incorporated in the DDBJ/EMBL/GenBank databases.

A prediction of the secondary structure model of the IVSs was carried out with mfold (www.bioinfo.rpi.edu/applications/mfold/srna.pl).

Total cellular RNA was extracted and purified from the *Campylobacter* cells using the RNAprotect bacteria reagent and RNeasy mini kit (Qiagen). The RNA was visualised by 1% (w/v) agarose gel electrophoresis in 1% (w/v) MOPS containing 2% (w/v) formaldehyde after heat denaturation of the total cellular RNA at 65°C for 15 min. The RNA was visualised by ethidium bromide staining.

Results

A PCR primer pair (fD1/r-*Ca*16h11) was used to amplify the helix 11 region in 16S rRNA gene sequences and amplicons were obtained from all isolates studied (Fig. 1). Sequencing

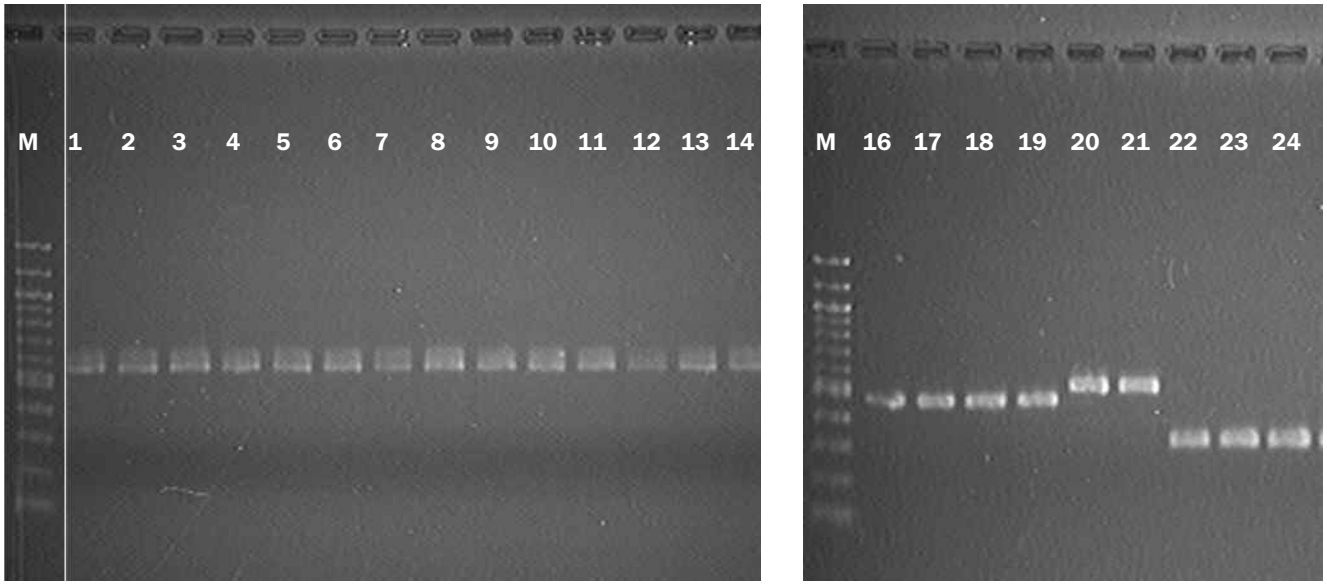

Fig. 1. Agarose gel electrophoresis profiles of PCR products amplified with *C. sputorum* and *C. curvus* isolates using the primer pair fD1 and r-*Ca*16h11. Lane M: 100-bp DNA ladder; lane 1: *C. sputorum* LMG7975; lane 2: LMG8535; lane 3: LMG10388; lane 4: LMG11765; lane 5: *C. sputorum* biovar *fecalis* LMG8531; lane 6: LMG8532; lane 7: LMG8534; lane 8: LMG6728; lane 9: LMG11761; lane 10: *C. sputorum* biovar paraureolyticus LMG17589; lane 11: LMG17590; lane 12: LMG17591; lane 13: LMG17592; lane 14: LMG17593; lane 16: *C. curvus* LMG7609; lane 17: LMG11033; lane 18: LMG11034; lane 19: LMG11127; lane 20: LMG11247; lane 21: LMG11249; lane 22: LMG13935; lane 23: *C. jejuni* 81–476; lane 24: *C. coli* JCM2529^T.

```
C. sputorum IVS 101:ATTGGGAAATG-TAGCTCTTAATAATATATATCAAAGATAATTTATAAATAAAAAGTTATAAATATAACAATAAGCTTTTTTGCAGCTTTATATAATAAA 199
C. curvus IVSA  101:.A.......GT--...C..ATG....G.T...TT...CA.T.AG..G..A.TT.GAC..A.CC..A.TTT..G.TT.---------------------- 175
C. curvus IVSB  101:.A.......GTCG.A.C..AT...T.G.T...TT.TC.A.T-------.CCT.CG.CGCA.CCG.A.GTTGCGGTG.---------------------- 170
C. jejuni       101:G.A......GT-------------------------------------------------------------------------------------- 111
C. coli         101:G.A......GT-------------------------------------------------------------------------------------- 111
                    ******

C. sputorum IVS 200:GCGAAAAAAAGTAAAGCAGTTAGATTTAATAAATTTTTATAGCATTTAAAAATACAAAAGACTTAATTTTTAAATCTAAATATAAATTATTACTAATATT 299
C. curvus IVSA  176:---------.TG.C.AGC.AAGCT.GG.G.G...C......A.T..AGGTGGGTGC.GG..GCG..AC.CCCGC..GT..AGACT.GCT.GG...G.C.G 266
C. curvus IVSB  171:----------------------------------------..T..AGGTGGGT...GG..GCGT.GC.CCTTG..GC..AGACGGACT..GT.CG.C.G 229
C. jejuni       111:-------------------------------------------------------------------------------------------------- 111
C. coli         111:-------------------------------------------------------------------------------------------------- 111


C. sputorum IVS 300:TTTAATAGTCATTTAGAAATATCTTAATATATATTAT----TAAGAGCTTTCGCTATGGGATGAGGCTATATTGTATCAGCTAGTTGG             383
C. curvus IVSA  267:CGA.G.C..A..A.TT..T.GCGAAGCA.GTA.A...TACA..G.GTT.C.....................C.............             354
C. curvus IVSB  230:CG------------------------A.GTA.A..ATATA..G.GTT.C.....................C.............             292
C. jejuni       112:-------------------------------------------------T.....G.G.A.......A......A.............             152
C. coli         112:-------------------------------------------------T.....G.G.A.......A......A.............             152
                                                                      * *** * * ******* ****** ***************
```

Fig. 2. Nucleotide sequence alignment analyses in the helix 11 region within 16S rRNA gene sequences. Numbers at the left and right refer to the nucleotide positions determined in the present study. Dots indicate identical bases, changes are indicated and dashes are deletions. Identical positions are marked by asterisks. *C. sputorum* IVS: all 13 *C. sputorum* isolates shown in Table 1; *C. curvus* IVSA, *C. curvus* LMG11247 and 11249; *C. curvus* IVSB, *C. curvus* LMG7609, 11033, 11034 and 11127.

and alignment analyses of the PCR amplicons of the helix 11 region showed that 14 *C. sputorum* (100%) and six *C. curvus* (86%) isolates carried IVSs in the helix 11 region, and no IVSs were found in *C. curvus* LMG13935 (Fig. 2).

An identical IVS sequence was identified in all 14 isolates of *C. sputorum* (AB501345; Fig. 2). In contrast, two IVSs containing distinct nucleotide lengths and sequences were found in the helix 11 regions from six *C. curvus* isolates (IVS A: *C. curvus* LMG11247 and 11249, AB501346; IVS B: *C. curvus* LMG 7609, 11033, 11034 and 11127, AB501347

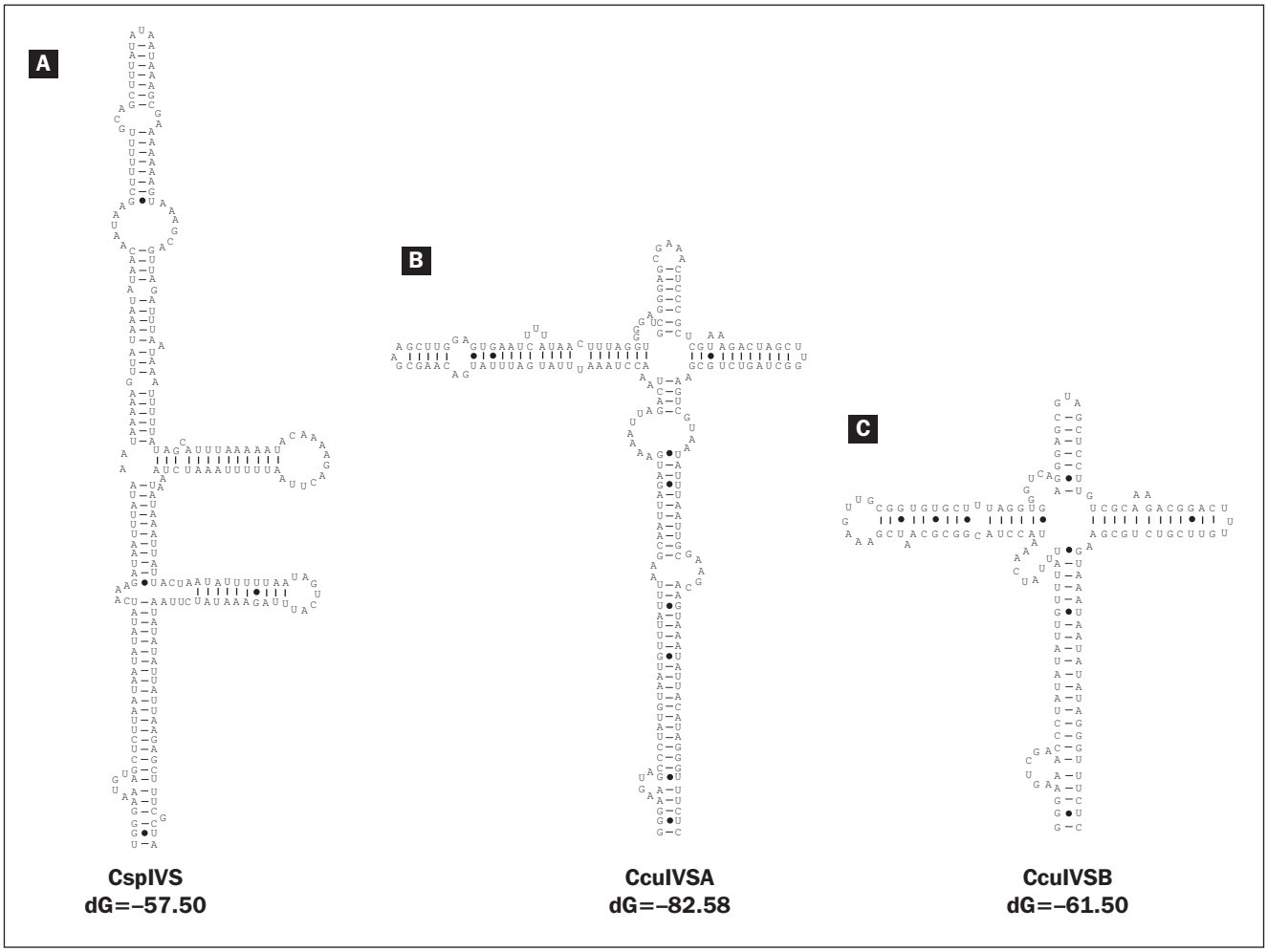

Fig. 3. Proposed secondary structures of IVSs in the helix 11 region within 16S rRNA genes. Secondary structure predictions were obtained using the mfold server available at bioinfo's home page.

[Fig. 2]). This suggests that different kinds of 16S rRNA gene are present in the *C. curvus* isolates.

Figure 3 shows the prediction of the secondary structure model of the IVSs in the helix 11 region of *C. sputorum* and *C. curvus*. In the present model, stem and loop structures were identified in the IVSs. Moreover, these are the first examples of the prediction of the secondary structure model of IVSs in 16S rRNA genes. Thus, two different secondary structure models of the IVSs from *C. curvus* were identified (Fig. 3).

As 16S rRNA molecules whose genes were longer in the helix 11 region were found to be fragmented in the three strains of *C. sputorum* (bv bubulus, fecalis and sputorum),[9] an attempt was made to perform denaturing agarose gel electrophoresis of the purified RNA fractions from the *C. sputorum* and *C. curvus* isolates. As shown in Figure 4, no 16S rRNA species were identified in the purified RNA fractions whose 16S rRNA genes carried IVSs. Instead of 16S rRNA, other smaller RNA fragments were identified. Thus, the primary 16S rRNA transcript may have been fragmented. The RNA purified from *C. curvus* LMG13935 cells, which did not contain IVSs, was employed as a reference marker.

Discussion

The authors have already identified four cases of *C. sputorum* bv sputorum LMG 7975 and bv fecalis LMG8531, LMG8534 and LMG6728 (n=4 *C. sputorum* bv sputorum; n=5 *C. sputorum* bv fecalis; n=5 *C. sputorum* bv paraureolyticus; n=7 *C. curvus*) to carry IVSs in the helix 25 region.[19] The reasons why *C. sputorum* and *C. curvus* carry IVSs in 16S and 23S rRNA genes so frequently are not known, but these two species may have frequent opportunities for those rRNA operons to interact with any sources of IVS. Moreover, they may have been able to integrate the IVSs into their own rRNA operons in the genome. Therefore, it may be worthwhile to identify other regions, such as the 16S-23S rRNA internal spacer region, the spacer region between 23S and 5S rRNA genes and the 5S rRNA structural gene, that may carry IVSs, and further research is in progress to resolve these questions.

Five *C. sputorum* isolates have already been reported to carry the IVSs in the helix 11 region within 16S rRNA genes.[9,13] Consequently, a total of 19 isolates of *C. sputorum* examined have been identified to carry IVSs. Thus, IVS integration in the 16S rRNA genes in the *C. sputorum* genome may precede differentiation into the three biovars (i.e., sputorum, fecalis and paraureolyticus).

At present, the reasons why these two species of *Campylobacter* frequently carry IVSs within 16S rRNA genes is not known. In the seven *C. curvus* isolates analysed both in the present study and in previous work,[17] one *C. curvus* isolate (LMG13935) lacked IVSs in all three helix regions within the 16S and 23S rRNA genes.

Natural transformation is a potential mechanism for horizontal gene transfer, leading to genetic diversity within a population.[20] It is suggested that intraspecies recombination plays a large part in producing genetic diversity among *C. jejuni* and *C. coli* strains.[20] Unfortunately, little is known about the transformation ability of *C. sputorum* and *C. curvus*; however, these two species may have a higher natural transformation ability to integrate IVSs into their own rRNA gene operons.

This is a first demonstration that atypical *C. sputorum* and *C. curvus* isolates frequently carry IVSs in the helix 11 region in 16S rRNA genes. In addition, these two species may have

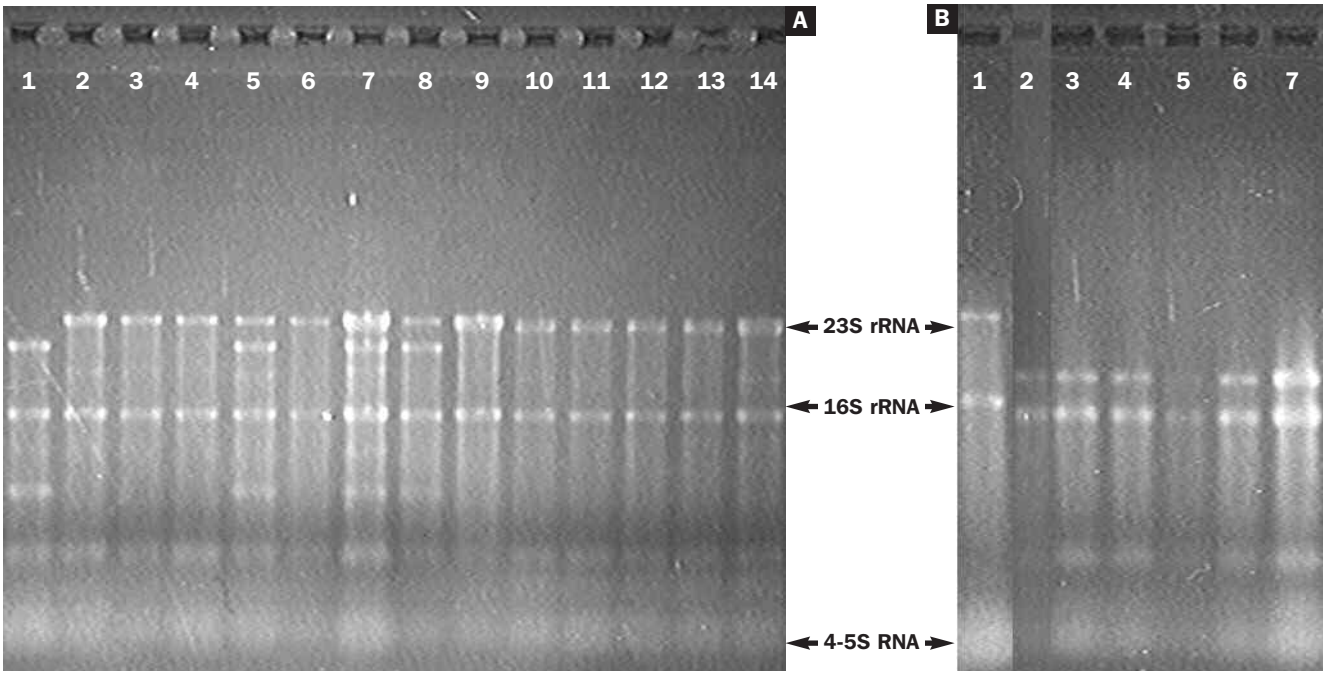

Fig. 4. Agarose gel electrophoresis of purified RNA from *C. sputorum* (A) and *C. curvus* (B) isolates containing IVSs in the helix region within 16S rRNA genes. A) Lane 1: *C. sputorum* bv sputorum LMG7975; lane 2: LMG8535; lane 3: LMG10388; lane 4: LMG11765; lane 5: *C. sputorum* bv fecalis LMG8531; lane 6: LMG8532; lane 7: LMG8534; lane 8: LMG6728; lane 9: LMG11761; lane 10: *C. sputorum* bv paraureolyticus LMG17589; lane 11: LMG17590; lane 12: LMG17591; lane 13: LMG17592; lane 14: LMG17593. B) Lane 1: *C. curvus* LMG13935; lane 2: LMG7609; lane 3: LMG11033; lane 4: LMG11034; lane 5: LMG11127; lane 6: LMG11247; lane 7: LMG11249.

a higher natural transformation ability to integrate IVSs into their rRNA gene operons, as well as IVSs in 23S rRNA genes. On the first prediction of the secondary structure model of the IVSs in 16S rRNA genes, stem and loop structures were identified; thus, the primary 16S rRNA transcripts may have been fragmented in the 20 isolates studied. □

This research was partially supported by The Promotion and Mutual Aid Corporation for Private Schools of Japan, Grant-in-Aid for Matching Fund Subsidy for Private Universities and by a Grant-in-Aid for Scientific Research (No. 20580346) to MM from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This study was also partially supported by a project grant (Start-Up Support for the Matching Fund Subsidy for Private Universities, 2007–2008) awarded by the Azabu University Research Services Division.

References

1 Edmonds P, Patton CM, Griffin PM *et al*. *Campylobacter hyointestinalis* associated with human gastrointestinal disease in the United States. *J Clin Microbiol* 1987; **25**: 685–91.

2 Martinot M, Jaulhac B, Moog R *et al. Campylobacter lari* bacteremia. *Clin Microbiol Infect* 2001; **7**: 96–7.

3 Patton CM, Shaffer N, Edmonds P *et al*. Human disease associated with '*Campylobacter upsaliensis*' (catalase-negative or weakly positive *Campylobacter* species) in the United States. *J Clin Microbiol* 1989; **27**: 66–73.

4 On SL, Atabay HI, Corry JE, Harrington CS, Vandamme P. Emended description of *Campylobacter sputorum* and revision of its infrasubspecific (biovar) divisions, including *C. sputorum* biovar paraureolyticus, a urease-producing variant from cattle and humans. *Int J Syst Bacteriol* 1998; **48**: 195–206.

5 Tanner ACR, Listgarten MA, Ebersole JL. *Wolinella curva* sp. nov: '*Vibrio succinogenes*' of human origin. *Int J Syst Bacteriol* 1984; **34**: 275–82.

6 Vandamme P, Falsen E, Rossau R *et al.* Revision of *Campylobacter, Helicobacter* and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int J Syst Bacteriol* 1991; **41**: 88–103.

7 Abbott SL, Waddington M, Lindquist D *et al*. Description of *Campylobacter curvus* and *C. curvus*-like strains associated with sporadic episodes of bloody gastroenteritis and Brainerd's diarrhea. *J Clin Microbiol* 2005; **43**: 585–8.

8 Wetsch NM, Somani K, Tyrrell GJ, Gebhart C, Bailey RJ, Taylor DE. *Campylobacter curvus*-associated hepatic abscesses: a case report. *J Clin Microbiol* 2006; **44**: 1909–11.

9 Van Camp G. Van De Peer Y, Nicolai S *et al*. Structure of 16S and 23S ribosomal RNA genes in *Campylobacter* species: phylogenetic analysis of the genus *Campylobacter* and presence of internal transcribed spacers. *Syst Appl Microbiol* 1993; **16**: 361–8.

10 Miller WL, Pabbaraju K, Sanderson KE. Fragmentation of 23S rRNA in strains of *Proteus* and *Providencia* results from intervening sequences (IVSs) in the rrn (rRNA) genes. *J Bacteriol* 2000; **182**: 1109–17.

11 Pronk LM, Sanderson KE. Intervening sequences in rrl genes and fragmentation of 23S rRNA in genera of the family Enterobacteriaceae. *J Bacteriol* 2001; **183**: 5782–7.

12 Linton D, Dewhirst FE, Clewley JP, Owen RJ, Burnens AP, Stanley J. Two types of 16S rRNA gene are found in *Campylobacter helveticus*: analysis, applications and characterization of the intervening sequence found in some strains. *Microbiology* 1994; **140**: 847–55.

13 Etoh Y, Yamamoto A, Goto N. Intervening sequences in 16S rRNA genes of *Campylobacter* sp.: diversity of nucleotide sequences and uniformity of location. *Microbiol Immunol* 1998; **42**: 241–3.

14 Harrington CS, On SL. Extensive 16S rRNA gene sequence diversity in *Campylobacter hyointestinalis* strains: taxonomic and allied implications. *Int J Syst Bacteriol* 1999; **49**: 1171–5.

15 Sambrook J, Russell DW. *Molecular cloning. A laboratory manual*. 3rd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2001.

16 Miyajima M, Matsuda M, Haga S, Kagawa S, Millar BC, Moore JE. Cloning and sequencing of 16S rDNA and 16S-23S rDNA internal spacer region (ISR) from urease-positive thermophilic *Campylobacter* (UPTC). *Lett Appl Microbiol* 2002; **34**: 287–9.

17 Sekizuka A, Tazumi A, Nakanishi S *et al*. Absence of intervening sequences (IVSs) in the helix 11 region within 16S rRNA genes among more than 240 isolates of the seven *Campylobacter* species. *Br J Biomed Sci* 2009; **66** (2): 103-6.

18 Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res* 1994; **22**: 4673–80.

19 Tazumi A, Kakinuma Y, Misawa N, Moore JE, Millar BC, Matsuda M. Identification and characterization of intervening sequences within 23S rRNA genes from more than 200 *Campylobacter* isolates from seven species including atypical campylobacters. *BMC Microbiol* 2009; **9**: 256.

20 Wiesner RS, Hendrixson DR, DiRita VJ. Natural transformation of *Campylobacter jejuni* requires components of a type II secretion system. *J Bacteriol* 2003; **185**: 5408–18.