Molecular characterisation of a type III restriction-modification system in *Campylobacter upsaliensis*

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ABSTRACT

Two examples of *Campylobacter upsaliensis* RM3195 and JV21 strains are shown to carry putative type III restriction (*res*)-modification (*mod*) enzyme gene clusters, following genome sequence analyses. It is suggested that the cluster is composed of at least three structural genes, *res*, internal methylase gene and *mod*, in the strains, based on the nucleotide sequence information. A ribosome binding site, a putative promoter consisting of a consensus sequence at the –10-like structure and a semiconserved T-rich region and a putative intrinsic ρ-independent transcriptional terminator were identified for the gene cluster in the two strains. Using two primer pairs, f-/r-res and f-/r-mod, 34 of 41 *C. upsaliensis* isolates generated two expected amplicons of the *res* and *mod* gene segments, and using another primer pair, the same number of isolates also generated an amplicon of the *res* and *mod* gene segments cluster, including the third internal methylase gene. Thus, *C. upsaliensis* isolates frequently carried putative type III R-M gene clusters, encoding the three enzymes. Interestingly, two possible overlaps were identified within the three tandem structural genes. In addition, the type III R-M gene cluster loci appear to be very similar among the *C. upsaliensis* isolates and very different from other thermophilic campylobacters.

KEY WORDS: Campylobacter upsaliensis.
High-throughput nucleotide sequencing.
Sequence analysis, DNA.
Type III R-M loci diversity.

Introduction

Restriction-modification (R-M) systems (type I,[1] type II,[2] type III,[3] and type IV[4]) occur in bacteria and are involved in protection of bacterial cells from invasion by foreign DNA. The type III R-M systems are composed of a restriction endonuclease that catalyses strand cleavage of unmethylated foreign DNA and a methyltransferase that performs the methylation of a specific DNA sequence.[5–7] Thus, the type III systems appear to consist of two subunits, namely restriction endonuclease (Res) and methyltransferase modification (Mod).[7] Recently, Ando *et al*. suggested that R-M systems may be associated with *Helicobacter pylori* virulence.[8]

Bacterial organisms within the genus *Campylobacter* are the most commonly recognised causes of acute bacterial diarrhoea in the Western world,[9–11] especially *Campylobacter jejuni*, *C. coli* and *C. lari*, which are the major and typically recognised *Campylobacter* organisms of medical, public health or veterinary interest worldwide.[12–14] *Campylobacter* enteritis is considered to be a zoonotic disease and domestic animals, such as poultry, cattle and pigs, can act as sources of infection.[13]

Campylobacter upsaliensis, a catalase-negative or weakly catalase-positive thermophilic *Campylobacter* species, was first isolated from faecal samples of healthy and diarrhoeic dogs in Sweden in 1983.[15] In addition, in 1989 this microorganism was obtained from cats.[16] Some descriptions concerning the risk to humans from these animals have appeared.[17,18]

Regarding type III R-M systems within the genus *Campylobacter*, seven strains (*C. jejuni* subsp. *jejuni* CG8486, DDBJ/EMBL/GenBank accession number NZ_AASY 00000000; *C. jejuni* RM1221, NC_003912; *C. jejuni* 81116, NC_009839; *C. jejuni* 414, CM000855; *C. jejuni* subsp. *doylei* 269.97, NC_009707; *C. upsaliensis* RM3195, NZ_AAFJ0000000; *C. upsaliensis* JV21, NZ_AEPU00000000) have already been shown to carry these systems among more than 20 *Campylobacter* strains whose complete whole genome or genome shotgun sequencings have been carried out, as described recently.[19,20] However, no detailed descriptions of the type III R-M enzyme genes have yet appeared for the genus *Campylobacter* strains.

Regarding type III R-M genes in the *C. upsaliensis* organisms, two human clinical *C. upsaliensis* strains, RM319520 and JV21, isolated from the faeces of a patient with Guillain-Barré syndrome and from a human gastrointestinal tract, respectively, were identified to carry type III R-M enzyme genes, following genome sequence analysis. In addition, no reports on type III R-M enzyme genes in *C. upsaliensis* organisms have yet appeared.

Therefore, the aim of the present study is to clarify whether or not the type III R-M enzyme genes or their homologue(s) occur in *C. upsaliensis* species isolates, and

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then molecularly characterise the type III R (*res*)-M (*mod*) enzyme gene loci from *C. upsaliensis* and compare these with other thermophilic campylobacters.

Materials and methods

Isolates and culture conditions

More than 40 isolates of *C. upsaliensis* species were analysed (Table 1). These isolates were cultured on Muller-Hinton agar (Oxoid, Hampshire, UK) containing 5% (v/v) defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) at 37˚C for 48 h in an aerobic jar under microaerophilic conditions.

Genomic DNA preparation

Genomic DNA was prepared from the *C. upsaliensis* cells using sodium dodecyl sulphate, proteinase K and phenol-chloroform extraction and ethanol precipitation.[21]

Primer design, PCR amplification and product purification

In a previous study, the authors constructed two primer pairs, f-/r-res and f-/r-mod, based on *C. upsaliensis* RM3195, *C. jejuni* subsp. *jejuni* CG8486 and *Helicobacter acinonychis* str. Sheeba (NC_008229) type III R-M gene sequence data in order to amplify the *res* and *mod* gene segments, respectively.[19] These two primer pairs were anticipated to generate polymerase chain reaction (PCR) products of the gene segments of approximately 1100 bp and approximately 700 bp, respectively.

Regarding the type III Res-Mod enzyme gene in the *C. upsaliensis* organisms, another internal methylase subunit gene has been shown to exist between *res* and *mod* genes in *C. upsaliensis* RM3195 and JV21, following their genome sequencing analyses (Fig. 1). The authors then designed another PCR primer pair, Cupres-F/CuplpxB-R, in order to amplify the res and mod gene segments, including the internal methylase subunit gene, in the present study (Fig. 2). These are anticipated to generate a PCR amplicon of approximately 2300–2400 bp in length with *C. upsaliensis*.

The PCR mixtures contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 400 µmol/L each dNTP, 1 µmol/L each primer, 50 ng each template genomic DNA and 1 unit GoTaq Colorless Master Mix (Promega, Tokyo, Japan). The PCR was carried out in a total of 25 µL reaction volumes at 95˚C for 5 min, for 30 cycles at 95˚C for 0.5 min, at 56.2˚C for 0.5 min, at 72˚C for 1.5 min and finally at 72˚C for 7 min.

Amplified products were then separated by 1% (w/v) agarose gel electrophoresis in 0.5×TBE at 100 V, detected by staining with ethidium bromide. The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan).

Cloning, nucleotide sequencing and sequence analyses

The purified PCR products were inserted into the pGEM-T vector with the pGEM-T Easy Vector System (Promega) using the TA cloning procedure. The reaction products were separated and detected on an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Tokyo, Japan), after dideoxy nucleotide sequencing using a Thermo Sequenase Pre-Mixed Cycle Sequencing Kit (Amersham Pharmacia Biotech, Tokyo, Japan).

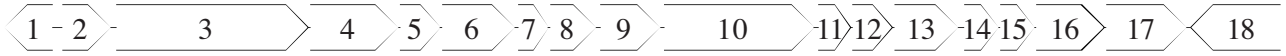
Table 1. *C. upsaliensis* isolates analysed in the present study, the summaries of the PCR analyses and the accession numbers of the type III R-M gene cluster loci.

No.	Isolate	Source	*res*	*mod*	R-M	Accession No.
1	Maliryn	Dog	+	+	+	AB736170
2	G1104	Dog	–	–	–	AB737974
3	12-1	Dog	+	+	+	AB736171
4	41-2	Dog	+	+	+	NA
5	29-3	Dog	–	–	–	AB736183, AB736184
6	13-1	Dog	+	+	+	NA
7	21-1	Dog	+	+	+	NA
8	42-3	Dog	+	+	+	NA
9	40-1	Dog	+	+	+	AB736172
10	26-4	Dog	+	+	+	AB736174
11	48-1	Dog	–	–	–	AB737975
12	49-1-1	Dog	+	+	+	NA
13	60-1	Dog	–	–	–	AB741647
14	66-1	Dog	+	+	+	NA
15	68-3	Dog	+	+	+	NA
16	70-3	Dog	–	–	–	AB741648
17	99-1	Dog	+	+	+	NA
18	101-1	Dog	+	+	+	NA
19	102-1	Dog	+	+	+	NA
20	105-1	Dog	+	+	+	NA
21	115-1	Dog	+	+	+	NA
22	feline 104-1	Cat	+	+	+	AB736175
23	feline 37-1	Cat	+	+	+	AB736176
24	2	Dog	+	+	+	NA
25	3	Dog	+	+	+	NA
26	4	Dog	+	+	+	AB736177
27	5	Dog	+	+	+	NA
28	6	Dog	+	+	+	AB736178
29	7	Dog	+	+	+	AB736179
30	8	Dog	+	+	+	AB736180
31	9	Dog	+	+	+	NA
32	11	Dog	+	+	+	AB736181
33	13	Dog	+	+	+	NA
34	14	Dog	+	+	+	AB736182
35	15	Dog	+	+	+	NA
36	16	Dog	+	+	+	NA
37	LMG8850	Dog	–	–	–	AB737976
38	G1	Dog	–	–	–	AB737977
39	CP01-03	NA	+	+	+	NA
40	CP03-314	NA	+	+	+	NA
41	12	Dog	+	+	+	NA

NA: not available.

Nucleotide sequence analyses were carried out using the Genetyx-Windows computer software version 9 (Genetyx, Tokyo, Japan). Nucleotide sequence alignment analyses to design the primer pairs were carried out using Clustal W software (1.7 program),[22] incorporated in the DDBJ.

A



B

No.	5'end (bp)	3'end (bp)	Gene	Product
1	77,950	77,180	*surE*	acid phosphatase SurE
2	78,057	78,797	–	conserved hypothetical protein
3	78,848	81,748	*res*	type III restriction enzyme R protein
4	81,738	83,096	–	type III restriction/modification enzyme, methylase subunit
5	83,096	83,722	*mod*	adenine specific DNA methylase
6	83,719	84,828	*lpxB*	lipid-A-disaccharide synthetase
7	84,838	85,323	*greA*	transcription elongation factor GreA
8	85,323	86,042	–	metallophosphoesterase
9	86,056	87,006	*cheV*	chemotaxis protein CheV
10	87,010	89,304	*cheA*	chemotaxis histidine kinase CheA
11	89,308	89,829	*cheW*	chemotaxis signal transduction protein CheW
12	89,829	90,452	*serB*	phosphoserine phosphatase
13	90,452	91,423	*tal*	transaldolase
14	91,481	92,017	*rplY*	50S ribosomal protein L25
15	92,014	92,559	*pth*	aminoacyl-tRNA hydrolase
16	92,562	93,614	–	integral membrane protein
17	93,608	94,834	*lysA*	diaminopimelate decarboxylase
18	96,241	94,862	*ftsA*	cell division protein FtsA

–, not available.

Fig. 1. Schematic representation of No. 1–18 genes containing the type III R (*res*) -M (*mod*) gene cluster identified in the *C. upsaliensis* RM3195 and JV21 strains (A) and the details of the No. 1–18 genes (B). The nucleotide positions used are for those of *C. upsaliensis* JV21 (NZ_AEPU00000000).

Results and discussion

As two examples of *C. upsaliensis* RM3195 and JV21 strains have been shown to carry the putative type III R-M enzyme genes, following genome sequence analyses, PCR amplifications for the *res* (No. 3, Fig. 1) and *mod* (No. 5, Fig. 1) genes were first performed using two primer pairs (f-/r-res and f-/r-mod) designed previously (Fig. 2).[19] When PCR was carried out with the 41 *C. upsaliensis* isolates (Table 1) using these primer pairs, 34 isolates generated two expected amplicons for the *res* and *mod* gene segments (Table 1, Fig. 3). The other seven isolates failed to generate any amplicons (Lanes 2, 5, 11, 13, 16, 37 and 38, Figs. 3A and 3B, and Table 1).

As schematically represented in Figures 1 and 2, it was suggested that the putative type III R-M gene cluster was composed of at least three structural genes (*res*, internal methylase subunit gene [No. 4, Fig. 1] and *mod*) in the *C. upsaliensis* isolates based on the type III R-M gene sequence data from the *C. upsaliensis* RM3195 and JV21 strains. Therefore, the putative type III R-M gene cluster and its adjacent genetic loci in the *C. upsaliensis* RM3195 and JV21 strains were then analysed.

Table 2. Summaries of the possible type III R enzyme, internal methylase subunit and M enzyme gene ORFs (bp) identified in the *C. upsaliensis* RM3195 and JV21 strains.

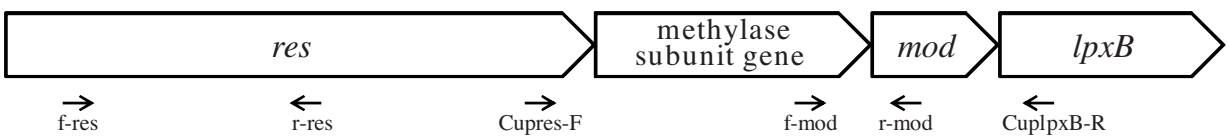
C. upsaliensis	R enzyme	methylase	M enzyme
RM3195	2,751	1,365	612
JV21	2,898	1,356	624

Possible type III R enzyme, internal methylase subunit and type III M enzyme gene open reading frames (ORFs) were identified in the two *C. upsaliensis* RM3195 and JV21 strains (Table 2). These were predicted to encode peptides of 917, 455 and 204 amino acid residues, with calculated molecular weights (CMWs) of 102, 50.6 and 22.8 kDa, respectively, for *C. upsaliensis* RM3195. For *C. upsaliensis* JV21, these three possible ORFs were also predicted to encode peptides of 966, 452 and 208 amino acid residues with CMWs of 107.4, 50.3 and 23.2 kDa, respectively.

Nucleotide sequence alignment analyses of approximately 70 bp regions immediately upstream of the conserved hypothetical protein gene (No. 2, Fig. 1) showed that the sequences were identical between the *C. upsaliensis* RM3195 and JV21 strains (Fig. 4A).

A probable ribosome-binding (RB) site (Shine-Dalgarno sequence),[23] ACGATG (nucleotide position [np] 78,051–78,056 bp for *C. upsaliensis* JV21) for the *res-mod* enzyme gene cluster, was identified, as well as the start codon ATG (np 78,057–78,059 bp), as shown in Figure 4A. Although a putative promoter consisting of consensus sequence at the –10-like region structure was also identified immediately upstream of the *res* genes within the two *C. upsaliensis* strains (Fig. 4A), no consensus sequence at the –35-like region was identified, and a semi-conserved T-rich

A



B

Primer	Nucleotide sequence (5'-3')
f-res	TTGTKCCAMGMAARGCVATYTTRG
r-res	ATTATCCCARCCYTCTTGYAAAGCCC
f-mod	GADGATGGMGTSATWTTYATMAGC
r-mod	ACNGCWTGNGCNRTNGTSCCRCTVCCKGC
Cupres-F	TTTGTCTGTGAGAGTAAGGG
CuplpxB-R	CGTGTGAGCTATAAAGAGGC

Fig. 2. Schematic representation of the type III R (*res*) -M (*mod*) gene cluster and adjacent genetic loci identified in the *C. upsaliensis* RM3195 and JV21 strains, including the locations of the novel PCR primers (A). Nucleotide sequences of the PCR primers are also shown (B).

region was identified between np 78,002 and 78,016 bp (T, 12/15). In addition, putative identical intrinsic ρ-independent transcriptional terminator structures, which contain a G+C-rich region near the base of the stem for the type III R-M enzyme gene cluster, were also identified in the two *C. upsaliensis* strains (Fig. 4B).

Thus, the type III R-M enzyme gene cluster and its adjacent genetic loci were shown to be very similar in *C. upsaliensis* RM3195 and JV21, and therefore an attempt was made to identify the putative type III R-M enzyme gene cluster following TA cloning and nucleotide sequencing of the PCR amplicon generated using the primer pair Cupres-F and CuplpxB-R (Fig. 2) with the *C. upsaliensis* Maliryn isolate used in the present study. Consequently, putative partial res, internal methylase subunit, mod and partial lpxB genes were identified in the *C. upsaliensis* Maliryn isolate

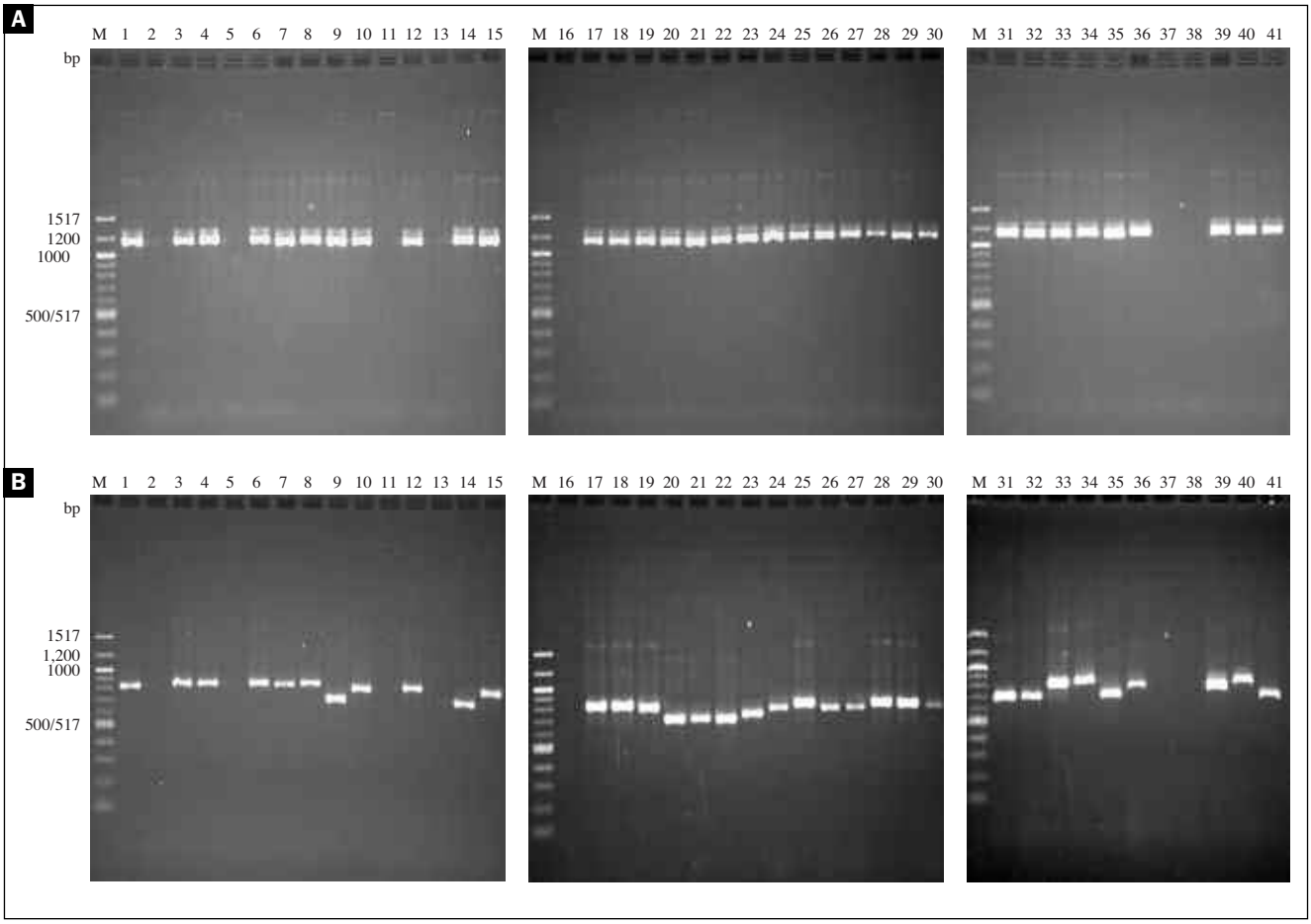


Fig. 3. Agarose gel electrophoresis profiles of PCR products of the *res* and *mod* gene segments amplified using two primer pairs of f-/r-res (A) and f-/r-mod (B) with 41 *C. upsaliensis* isolates (Lanes 1–41, following the order of the isolate numbers shown in Table 1). Lane M: 1 kbp DNA ladder (New England BioLabs Japan, Tokyo, Japan).

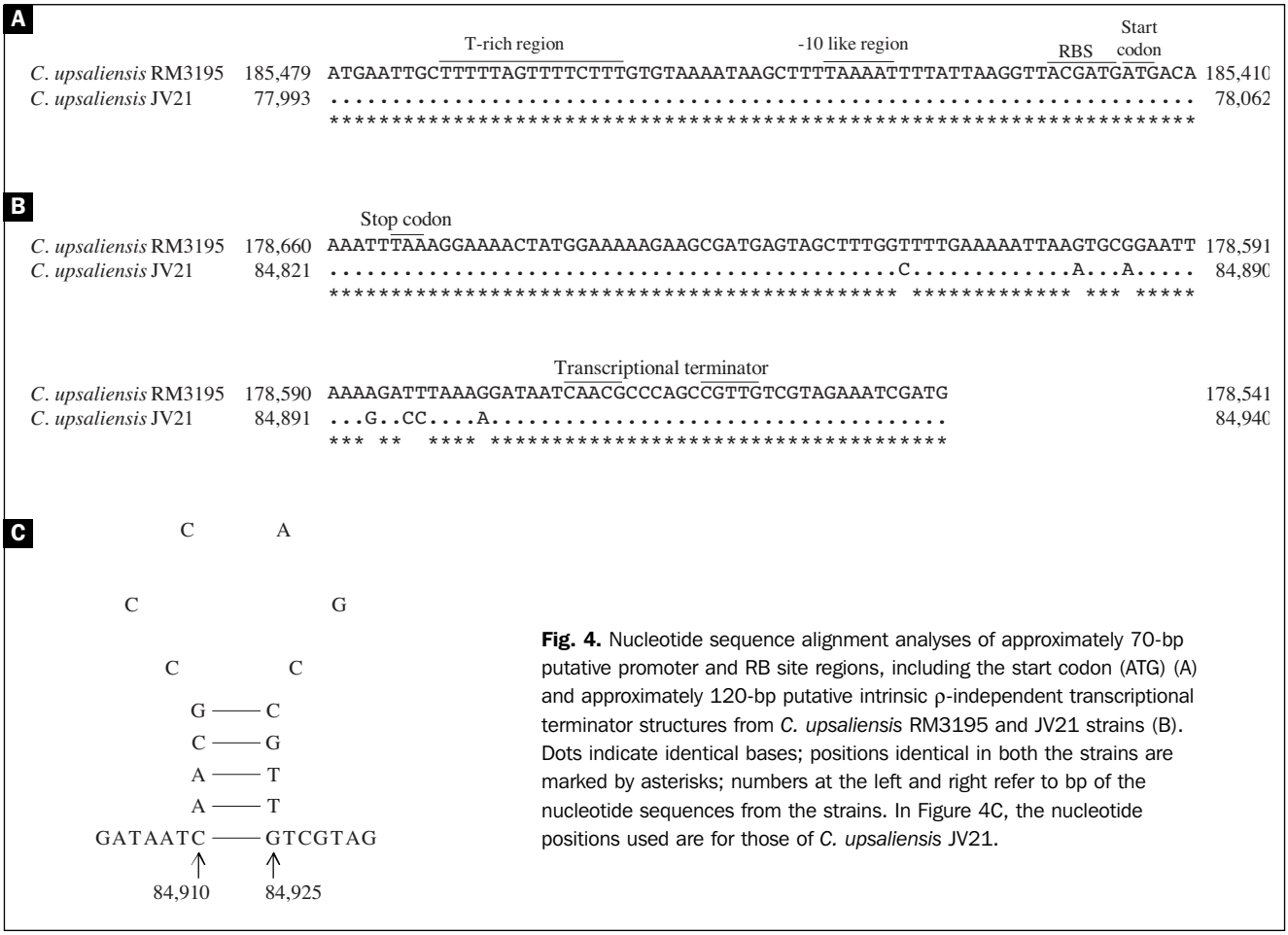


Fig. 4. Nucleotide sequence alignment analyses of approximately 70-bp putative promoter and RB site regions, including the start codon (ATG) (A) and approximately 120-bp putative intrinsic ρ-independent transcriptional terminator structures from *C. upsaliensis* RM3195 and JV21 strains (B). Dots indicate identical bases; positions identical in both the strains are marked by asterisks; numbers at the left and right refer to bp of the nucleotide sequences from the strains. In Figure 4C, the nucleotide positions used are for those of *C. upsaliensis* JV21.

(AB736170, Table 1), based on the comparisons of nucleotide and deduced amino acid sequence similarities with those of the type III corresponding genes from the *C. upsaliensis* RM3195 and JV21 strains.

Interestingly, two possible overlaps were identified between the *res* and the internal methylase subunit genes and between the internal methylase subunit and *mod* genes in both the *C. upsaliensis* RM3195 and JV21 strains and also in the *C. upsaliensis* Maliryn isolate, respectively. Thus, the type III R-M enzyme gene cluster and its adjacent genetic loci appear to be very similar among these three *C. upsaliensis* isolates.

Amplification was then carried out using the Cupres-F/CuplpxB-R primer pair (Fig. 2) designed *in silico* in order to clarify whether or not the internal methylase subunit gene exists between the *res* and the *mod* genes in *C. upsaliensis* isolates, as described above. When PCR was carried out with 40 *C. upsaliensis* isolates (Lanes 2 to 41, Fig. 5) using the primer pair, 33 isolates generated the expected amplicons of the segments (approximately 2300–2400 bp in length), as

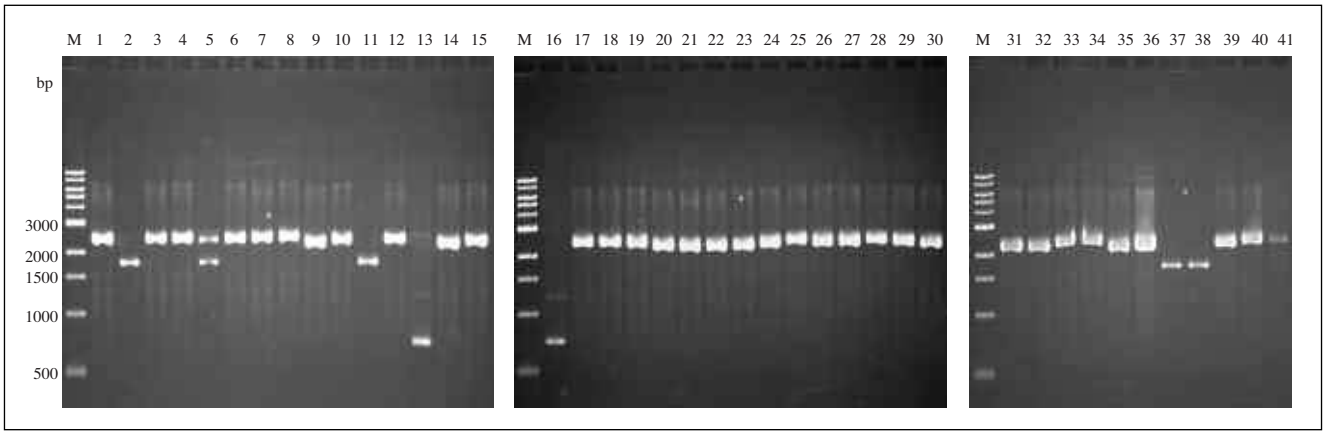


Fig. 5. Agarose gel electrophoresis profiles of PCR products of the *res* and *mod* gene cluster segments including an internal methylase subunit gene amplified using the primer pair Cupres-F and CuplpxB-R with 41 *C. upsaliensis* isolates. Lane M: 1 kbp DNA ladder (New England BioLabs Japan.). For Lanes 1–41, refer to the legend to Figure 3.

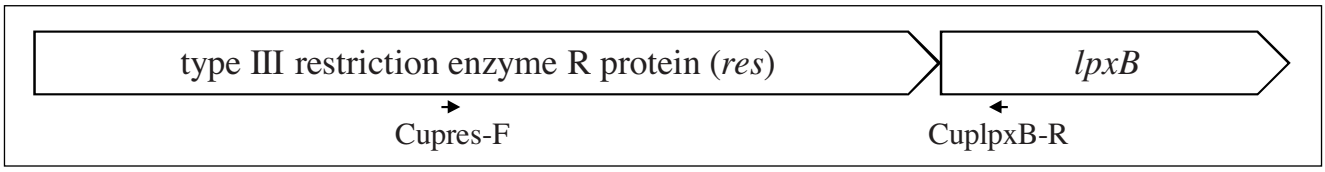


Fig. 6. Schematic representation of an atypical *res-lpxB* gene locus within the 1800-bp amplicon generated using the primer pair Cupres-F/CuplpxB-R, with five *C. upsaliensis* isolates.

well as the *C. upsaliensis* Maliryn isolate (Fig. 5). The other seven isolates generated three different amplicon sizes (Lane 2, *C. upsaliensis* G1104 [approximately 1800 bp]; lane 5, 29-3 [approximately 2300–2400 bp and 1800 bp]; lane 11, 48-1 [approximately 1800 bp]; lane 13, 60-1 [approximately 750 bp]; lane 16, 70-3 [approximately 750 bp]; lane 37, LMG8850 [approximately 1800 bp]; lane 38, G1 [approximately 1800 bp]). The results of the PCR amplicon profiles (R-M) are summarised in Table 1.

These results indicate that *C. upsaliensis* organisms carry the R-M enzyme genes, including an internal methylase subunit gene within their genomic DNA with a frequency of approximate 83%.

The present study describes three overlapping genes of a res gene, an internal methylase subunit gene and a mod gene as the putative type III R-M enzyme system genes in the two *C. upsaliensis* RM3195 and JV21 strains. These similar profiles of the type III R-M enzyme genes were also found in the *C. upsaliensis* Maliryn isolate (AB736170). As shown in Figure 5, the other 33 *C. upsaliensis* isolates generated an approximate 2300–2400 bp amplicon, suggesting an occurrence of the internal methylase subunit gene between the *res* and *mod* genes, using the primer pair Cupres-F/CuplpxB-R (Fig. 5, Table 1).

In addition, the five *C. upsaliensis* isolates of G1104, 29-3, 48-1, LMG8850 and G1 generated an approximate 1800 bp amplicon, and the 60-1 and 70-3 isolates generated an approximate 750 bp amplicon (Fig. 5). Thus, the three different sizes of amplicon, approximately 2300–2400 bp, 1800 bp and 750 bp, were generated in the PCR experiments using the primer pair Cupres-F/CuplpxB-R with *C. upsaliensis* isolates.

An attempt was then made to sequence these amplicons (approximately 1800 bp and 750 bp) generated in the seven *C. upsaliensis* isolates. The five C. *upsaliensis* isolates that generated the 1800 bp amplicons carried an atypical *res-lpxB* gene locus (Fig. 6). A schematic profile (Fig. 6) suggests that the atypical locus may be generated by the replacement of the *C. upsaliensis res* gene with the *C. jejuni res* gene and deletion of the internal methylase subunit gene-mod gene segment, based on the nucleotide sequence alignment analyses with *C. upsaliensis* G1104, RM3195 isolates and the *C. jejuni* subsp. *doylei* 269.97 strain (NC_009707) (data not shown). The *C. upsaliensis* 29-3 isolate, which carried the two amplicons of approximately 2300–2400 bp and 1800 bp generated in the PCR experiment using the primer pair Cupres-F/CuplpxB-R, carried both the R-M enzyme gene cluster, including an internal methylase subunit gene, and the atypical *res-lpxB* gene locus (AB736183, AB736184, Table 1). In addition, the 750-bp amplicon, generated with the *C. upsaliensis* 60-1 and 70-3 isolates, was shown not to fit any known nucleotide sequences using BLAST analysis (data not shown).

The R-M systems have been classified into four distinct types, type I,[1] type II,[2] type III[3] and type IV,[4] based on subunit compositions, cofactor requirements and modes of DNA cleavage. Among them, in general, the type III R-M systems appear to be composed of a restriction endonuclease and a methyltransferase.[5–7] In addition, Su *et al*. showed that the type III R-M systems require at least two functional genes, *res* and *mod*.[24]

Overall, all the type III R-M enzyme systems in the thermophilic *Campylobacter*, *C. jejuni* and *C. lari* organisms, whose complete whole genome or genome shotgun sequencing have been elucidated or type III R-M systems reported,[19] are composed of a restriction endonuclease (Res) and a methyltransferase (Mod) encoded on *res* and *mod* genes, respectively.

Regarding *C. coli*, no type III R-M system has been shown to exist. For example, *C. coli* RM2228 isolated from chicken had been

		Ado-Met binding site	
UPTC CF89-12 mod	112	YYEKIKMIYIDPPYNTKNDKFI	133
C. jejuni CG8486 mod	113	.....	134
C. upsaliensis RM3195	113	.DG.....	134
C. upsaliensis JV21	113	.DG.....E...	134
C. upsaliensis Maliryn	113	.D.....E...	134
C. upsaliensis 4	113	.D.....SE...	134
C. upsaliensis 6	113	.D.....E...	134
C. upsaliensis 7	113	.D.....SE...	134
C. upsaliensis 8	113	.D.....SE...	134
C. upsaliensis 11	113	.D.....E...	134
C. upsaliensis 12-1	113	.D.....E...	134
C. upsaliensis 14	119	.D.....E...	140
C. upsaliensis 26-4	119	.D.....E...	140
C. upsaliensis 29-3	113	.D.....E...	134
C. upsaliensis 40-1	113	.D.....D...E...	134
C. upsaliensis feline 37-1	113	.D.....E...	134
C. upsaliensis feline 104-1	113	.D.....E...	134
*Hin*fIII[25]	139	FKG.V.L.....G..G.K	160
*Lla*FI[25]	191	.A...QC.....S..	212
*Eco*P15[25]	113	.A.....K...V	134
*Eco*P1[25]	113	.A...N.....K...V	134
*Sty*LT[25]	135	.ADT.D.....S...V	146
		******* * *	

Fig. 7. Deduced amino acid sequence multiple alignment analysis of the N-terminal regions of the putative M enzyme ORF from 16 *C. upsaliensis* isolates, as well as those from other bacterial organisms already described.[24] Dot indicates identical amino acid residues; changes are indicated; identical positions in all cases are marked by asterisks. The numbers at the left and right refer to the positions of each amino acid residue of the putative M enzyme ORFs. The Ado-Met binding site (DPPY) is highlighted.

sequenced by the random shotgun method and its genome (NZ_AAFL00000000)[20] was shown not to carry any type III R-M enzyme systems.

Although the human clinical *C. lari* RM2100 strain has been shown not to carry any type III R-M systems (NC_012039), a R-M gene cluster was first found downstream of the full-length cytolethal distending toxin gene operon in the urease-positive thermophilic *Campylobacter* (UPTC) CF89-12 strain within the *C. lari* species.[19,25] In the UPTC strain, two putative ORFs for the restriction endonuclease and the adenine-specific DNA methyltransferase were predicted to encode peptides of 947 and 613 amino acid residues with CMWs of 111 and 70.8 kDa, respectively.[19]

When PCR was carried out with the 16 *C. lari* isolates (UPTC [*n*=9], urease-negative [UN] *C. lari* [*n*=7]) using the primer pair f-/r-res and f-/r-mod, six UPTC and two UN *C. lari* isolates generated two expected amplicons for a *res* and a *mod* gene segment, as already described.[19] In addition, one UPTC isolate produced only the *mod* gene segment and the other seven *C. lari* isolates failed to generate any amplicons.[19] Thus, UPTC isolates were identified to carry the type III R-M enzyme genes with a relatively high frequency.[19] However, these are very different from the other thermophilic *Campylobacter* organisms carrying the type III enzyme system with low frequency.[19,25]

In a previous study, Nakajima *et al*. found a putative Ado-Med binding motif in the N-terminal region of the M enzyme from the UPTC CF89-12.[19] In the present study, the authors also identified the Ado-Met binding motif (DPPY) in the N-terminal region of the mod enzyme from the *C. upsaliensis* isolates examined (Fig. 7).

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