Molecular and comparative analyses of the full-length cytolethal distending toxin (*cdt*) gene operon and its adjacent genetic loci from urease-positive thermophilic *Campylobacter* (UPTC) organisms

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ABSTRACT

Molecular and comparative analyses of the full-length cytolethal distending toxin (*cdt*) gene operon and its adjacent genetic loci (2.7–9.4 kilo base pairs in length) are carried out with 12 urease-positive thermophilic *Campylobacter* (UPTC) isolates using several polymerase chain reaction (PCR) primer pairs. Three putative open reading frames (ORFs) for *cdtA, cdtB* and *cdtC*, two putative promoters and a hypothetically intrinsic ρ-independent transcription terminator were identified in all the operons of the 12 UPTC isolates examined. Although the number of amino acid residues slightly varied for the putative *cdtA* and *cdtC* ORFs, those for the *cdtB* were similar among all the UPTC isolates, as well as the six urease-negative (UN) *C. lari* examined previously. Regarding the *cdt* genes in UPTC CF89-12, each ORF commenced with an ATG start codon and terminated with a TAG stop codon for *cdtA* and *cdtB* and a TAA for *cdtC*. Start and stop codons of the three ORFs for the other 11 UPTC isolates were identical to those from the UPTC CF89-12 isolate except for the TTG start codon for *cdtC* in the two isolates (NCTC12892 and 12893) and the TGA stop codon for *cdtA* in five isolates (A1, A2, A3, 89049 and 92251). Two putative promoter structures, consisting of sequences at the –35-like (TTAATA) and –10-like (TATTAA) regions, as well as the start codon (ATG), were identified for the transcriptional promoter, immediately upstream of the *cdtA* gene in all the 12 isolates, Although the genetic heterogeneity of the *cdtB* gene locus occurred in all 28 *C. lari* isolates (n=16 UN *C. lari*; n=12 UPTC) examined, all nine amino acid-specific DNase residues were completely conserved in all their *cdtB* genes. Variable gene insertions with heterogeneous order and combinations occurred between *cdtC* and *lpxB* genes in the all UPTC organisms examined.

KEY WORDS: Base sequence.
Campylobacter.
Genes.
Genetics, molecular.

Introduction

Thermophilic *Campylobacter* species, particularly *C. jejuni* and *C. coli,* are the recognised causes of acute bacterial diarrhoea worldwide. *C. lari* is an atypical thermophilic *Campylobacter* species consisting of at least two representative taxa, urease-negative (UN) *C. lari* and urease-positive thermophilic *Campylobacter* (UPTC).[1,2]

Regarding campylobacteriosis, there remains a general absence of information about the mechanisms involved in the pathogenesis of infection. Although many descriptions of the virulence factors for campylobacteriosis have been carried out,[3] very little is known about *Campylobacter* pathogenesis.

Among several *Campylobacter* cytotoxins already identified,[4,5] the cytolethal distending toxin (CDT) has been characterised in detail.[6–8] The *cdt* genes of *C. jejuni* have been cloned, sequenced and characterised.[6] CDT causes progressive cellular distension and ultimately death in several cell lines,[9,10] and, moreover, CDT causes cell cycle blockage.[9]

Regarding CDT, it is suggested that CDTB is a toxic component (deoxyribonuclease) and that CDTA and C are carriers of CDTB to target cells.[11] In addition, Asakura *et al.* recently reported comparative analysis of *cdt* genes among typical *C. jejuni, C. coli* and *C. fetus* strains.[12]

This research group has already demonstrated genetic heterogeneity of the *cdtB* gene of *C. lari* isolates after polymerase chain reaction (PCR) cloning, sequencing and analysis of the *cdtB* gene fragments (approximately 720 base pairs [bp] in length) from 24 *C. lari* isolates, including eight UPTC isolates.[13] Moreover, the authors have recently described the full-length *cdt* genes, including the putative promoter and terminator structures from UN *C. lari* isolates and the comparison of their molecular characteristics with those of other thermophilic campylobacters, in order to clarify the molecular entities of the full-length *cdt* gene

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operon within *C. lari* organisms, using the primer pair of f-/r-Cl*cdt*all.[14] However, no PCR amplicons could be generated with the UPTC isolates, although an attempt was made to amplify the corresponding segment using the primer pair with the genomic DNAs from at least 10 UPTC isolates. Therefore, new approaches might have been required to analyse the full-length *cdt* genes from UPTC organisms.

Although, to date, cases of human illness associated with UN *C. lari* have been infrequent, clinical isolates from patients have added to approximate 80 isolates from about 20 cases in several countries over the past 25 years.[15–18] Although, in addition, four human isolates of UPTC were obtained in France in the period from 1986 to 1989,[19,20] any possible association of urease-producing UPTC with human diseases remains unclear. Therefore, it would be worthwhile to clarify whether or not UPTC organisms carry the *cdt* gene operon, one of the representative virulent genes.

The aim of the present study is to analyse the full-length *cdt* gene operon and its adjacent genetic loci from UPTC organisms and compare their molecular characteristics to each other and to those from UN *C. lari* and *C. jejuni* isolates.

Materials and methods

Twelve isolates of UPTC obtained from different sources in various countries were analysed in the present study, as shown in Table 1. The cells were cultured on Mueller-Hinton broth that contained 7% (v/v) defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) supplemented with *Campylobacter*-selective medium (Nissui, Tokyo, Japan), under microaerophilic conditions at 37°C for 48 h.[21] An atmosphere of 5% (v/v) O_2 and 10% (v/v) CO_2 was produced by BBL Campypak Microaerophilic System Envelopes (Becton Dickinson, NJ. USA).

Template DNA was prepared using sodium dodecyl sulphate and proteinase K treatment, phenol-chloroform extraction and ethanol precipitation,[22] and adjusted to approximately 500 ng/μL. Figure 1 shows a schematic representation of the *cdt* gene operon and the genetic loci for the UPTC CF89-12 organism (DDBJ/EMBL/GenBank Accession No. AB509347) analysed in the present study. Also designed were several kinds of PCR primer pairs *in silico* for amplification of the *cdt* gene operon from the UPTC CF89-12 isolate (Fig. 1).

The present study also included inverse (I)-PCR with the completely digested genomic DNAs from the UPTC CF89-12 isolate with *Bgl* II, *Hha* I, *PshB* I and *Msp* I, respectively (Fig. 1). The primer pairs for the I-PCR were partially constructed based on the nucleotide sequence data of the partial *cdtB* gene fragments (approximately 720 bp) from the UPTC CF89-12 (Fig. 1).[13]

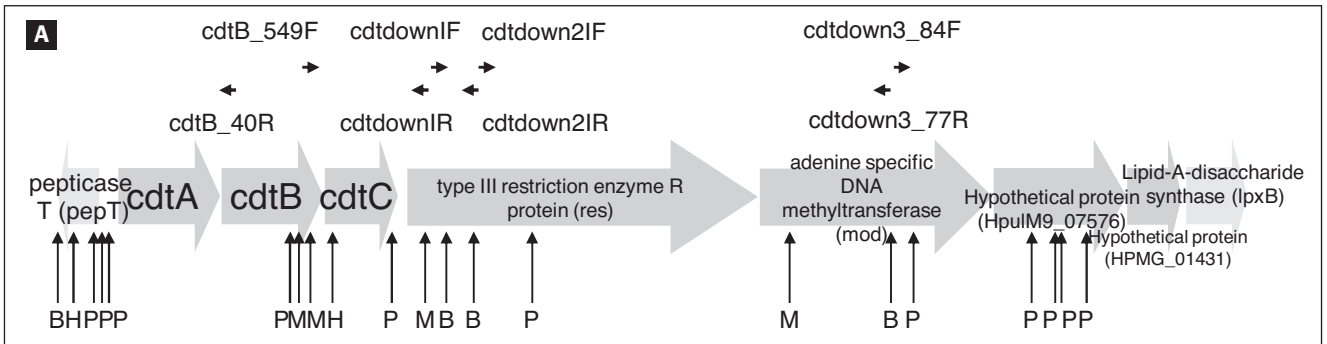
The PCR mixture contained 1x iProof HF buffer, 200 μmol/L each dNTP, 0.5 μmol/L each primer (Fig. 2), a total of 1 unit iProof DNA polymerase (Bio-Rad Laboratories, Tokyo, Japan) and 1 μL template DNA. The PCR reaction was performed in 50 μL reaction volumes at 98°C for 30 sec, with 35 cycles of 98°C for 5 sec, 50°C for 10 sec, and 72°C for 10 sec to 3 min, as shown in parentheses in Figure 2A, followed by a final extension of 72°C for 5 min.

Then, PCR primer pairs were constructed for

Table 1. UPTC and UN *C. lari* isolates and two *C. jejuni* reference strains analysed in the present study, with accession numbers of the nucleotide sequence data of the *cdt* gene operon including adjacent genetic loci accessible in the DDBJ/EMBL/GenBank.

Campylobacter	Isolate no.	Source	Country	Accession number
UPTC	CF89-12	River water	Japan	AB509347
UPTC	CF89-14	River water	Japan	AB509348
UPTC	NCTC12892	River water	England	AB509349
UPTC	NCTC12893	River water	England	AB509350
UPTC	NCTC12894	Sea water	England	AB509351
UPTC	NCTC12895	Mussel	England	AB509352
UPTC	NCTC12896	Mussel	England	AB509353
UPTC	A1	Seagull	N. Ireland	AB509354
UPTC	A2	Seagull	N. Ireland	AB509355
UPTC	A3	Seagull	N. Ireland	AB509356
UPTC	89049	Human	France	AB509357
UPTC	92251	Human	France	AB509358
UN *C. lari*	JCM2530T	Seagull	Japan	AB292351
UN *C. lari*	264	Mussel	Japan	AB292354
UN *C. lari*	298	Human	Japan	AB292355
UN *C. lari*	84C-1	Human	N. Ireland	AB292352
UN *C. lari*	84C-2	Mussel	N. Ireland	AB292353
UN *C. lari*	448	Mussel	N. Ireland	AB292356
UN *C. lari*	RM2100	Human	USA	AAFK01000004
C. jejuni	RM1221	Chicken	USA	NC_003912
C. jejuni	NCTC11186	Human	USA	NC_002163

UPTC, urease-positive thermophilic *Campylobacter*; UN, urease-negative; JCM, Japan Collection of Microorganisms; N. Northern.



B

Primer	Nucleotide sequence (5'→3')
cdtB_549F	TAATATAGTATCACCGCC
cdtB_40R	TACTTTCAGTTGATGCTG
cdtdownIF	TTAATGGAAACAGGCACAGG
cdtdownIR	CTAAATTTAACTTATCGGTG
cdtdown2IF	GATGAGCCACATTTGC
cdtdown2IR	TATGATACTAATAGGC
cdtdown3_84F	CTTGTGGAACAATGTGGCAC
cdtdown3_77R	CCTGTCTAACCTCACTAAG

Fig. 1. A schematic representation of the *cdt* gene operon and its adjacent genetic loci in the UPTC CF89-12 genome DNA, and its restriction map constructed with the restriction enzymes, *Bgl* II (B), *Hha* I (H), *PshB* I (P) and *Msp* I (M) following the I-PCR direct sequencing and analysis procedure. (A) *pepT*, peptidase T, *lpxB*, lipid-A-disaccharide synthase. (B) Some primer pairs and their nucleotide sequences designed *in silico* for the I-PCR with the completely designed genetic DNAs from the UPTC CF89-12 with the four restriction enzymes.

amplification of the full-length *cdt* gene operons and their adjacent genetic loci from 11 other UPTC isolates, as shown in Figure 2. Regarding the regions downstream of *cdtC* gene, other PCR and I-PCR primer pairs were also constructed (Fig. 2). Also employed was a primer pair of 128946_*cdt*CF and *lpxB*_196R to amplify the regions downstream of the *cdtC* gene from some UPTC isolates by PCR procedure (Fig. 2). In this case, the PCR mixture contained 1xPCR buffer for KOD-Plus (Toyobo, Osaka, Japan), 1.0 mmol/L $MgSO_4$, 200 μmol/L each dNTP, 0.3 μmol/L each primer, 50 ng template DNA and a total of 1 unit KOD-Plus (Toyobo). The PCR method was performed in 50 μL reaction volumes at 96°C for 2 min, with 35 cycles of 96°C for 20 sec, 50°C for 30 sec and 68°C for 10 min, followed by a final extension of 68°C for 10 min.

Amplified PCR products were separated by 0.7% (w/v) agarose gel electrophoresis in 0.5xTBE at 100 V and detected by staining with ethidium bromide. The PCR products amplified by the newly constructed primer pairs for the *cdt* gene operon were purified using a QIAEXII gel extraction kit (Qiagen, Tokyo, Japan). The purified amplicons were then subjected to cycle sequencing with BigDye Terminator (Applied Biosystems, Tokyo, Japan), with the PCR primers or the I-PCR primers and other sequence primers constructed by primer walking procedures (data not shown). The reaction products were separated and detected on an ABI 310 genetic analyser (Applied Biosystems). Sequence analysis of the full-length *cdt* gene operon was carried out using the GENETYX-Windows computer software (version 9, GENETYX, Tokyo, Japan). Nucleotide and deduced amino acid sequences of the *cdt* gene operon and its adjacent genetic loci from the 12 UPTC isolates examined, as well as the six UN *C. lari* isolates determined previously,[14] were compared to each other and to accessible sequence data from the *C. jejuni* isolates using CLUSTAL W software, which was incorporated in the DDBJ.[23] In the present study, of the six UN *C. lari* isolates whose *cdt* gene operons have already been described, 14 were further analysed comparatively with their *cdt* gene information from *C. jejuni* organisms accessible in DDBJ/EMBL/GenBank.

Results

Sequencing and analysis of the full-length *cdt* gene operon from the 12 UPTC isolates whose PCR amplicons could not be generated previously[14] were examined. Nucleotide sequences containing the full-length *cdt* gene operon from the 12 UPTC isolates determined in the present study are accessible in the DDBJ/EMBL/GenBank, as shown in Table 1.

Following analysis, sequences of approximately 9.4 kbp were identified encoding a *cdt* gene operon, including two putative promoter structures and a hypothetically ρ-independent intrinsic transcription terminator of approximately 2.35 kbp, and partial and putative open reading frames (ORFs) immediately upstream and downstream of the operon from the UPTC CF89-12. With UPTC CF89-12, the present sequence analysis identified three putative ORFs, including an 804 bp (nucleotide positions [np] 480–1283 for UPTC CF89-12) for *cdtA*, 804 bp (np 1296–2099 bp) for *cdtB* and 576 bp (np 2112–2687 bp) for *cdtC*, all transcribed and translated in the same direction. In

the present study, the nucleotide positions used are those of the UPTC CF89-12 isolate. In Table 2 the summaries of the three ORFs, *cdtA, cdtB* and *cdtC*, from the other 11 UPTC isolates examined are shown. The putative ORFs from the 12 isolates were predicted to encode peptides of different numbers of amino acid residues for *cdtA* and *cdtC*, as shown in Table 2. However, amino acid residue numbers were similar (n=268) for *cdtB* among the 18 *C. lari* isolates, including the six UN *C. lari* isolates previously examined.[14] In the present study, three putative ORFs of *cdtA, cdtB* and *cdtC* were identified, based on comparison of nucleotide and deduced amino acid sequence similarities with those of the corresponding *cdt* genes from UN *C. lari* isolates.[14] Calculated molecular weights (CMWs) of *cdtA, cdtB* and *cdtC* from all 12 UPTC isolates were shown to be relatively similar to each other and to those of UN *C. lari* and *C. jejuni* strains (Table 2).[6,14,24]

With regard to the *cdt* genes in UPTC CF89-12, each ORF commenced with an ATG start codon and terminated with a TAG stop codon for *cdtA* and *cdtB*, and a TAA for *cdtC*. Start and stop codons of the three ORFs for the other 11 UPTC isolates were identical to those from the UPTC CF89-12 isolate, except for the TTG start codon for the *cdtC* in the two UPTC isolates (NCTC12892 and 12893) and the TGA stop codon for the *cdtA* in the five UPTC isolates (A1, A2, A3, 89049 and 92251). Interestingly, a possible nucleotide overlap was identified between A of the stop codon (TGA) for the *cdtA* and A of the start codon (ATG) for the *cdtB* in the five isolates (A1, A2, A3, 89049 and 92251). This research group had already described a possible overlap among four nucleotides (ATGA), including the stop codon (TGA) of *cdtA* and the start codon (ATG) of *cdtB* in all the six UN *C. lari* isolates examined.[14] In addition, it has also been reported that the *cdtA* and *cdtB* genes have a four-nucleotide overlap in the *C. jejuni* 81–176 strain.[7]

Two putative promoter structures, consisting of sequences at the –35-like (TTAATA; np 385–390 bp) and –10-like (TATTAA; np 405–410 bp) regions, as well as the start codon (ATG; np 480–482 bp), were identified for the transcriptional promoter immediately upstream of the *cdtA* gene in all 12 isolates, as shown in Figure 3. In Figure 3, similar promoter structures were also identified to occur for the other 11 UPTC isolates.

Probable ribosome-binding (RB) sites (Shine-Dalgarno [SD] sequences)[25,26] that are complementary to a highly conserved sequence of CCUCCU, close to the 3′ end of 16S ribosomal RNA, AGGAGG (np 470–475 bp) for *cdtA* (Fig. 3), AGGAG (np 1285–1289 bp) for *cdtB* and AGGAG (np 2101–2105 bp) for *cdtC* and a hypothetically ρ-independent intrinsic transcriptional terminator sequence (np 2697–2710 bp) were identified with UPTC CF89-12 (Fig. 4A). The hypothetically intrinsic ρ-independent transcription terminator structure, which contains a G+C-rich region near the base of the stem, was identified immediately downstream of *cdtC* (Fig. 4B). The start codons of the three *cdt* genes were preceded by SD sequences. Thus, the *cdtA, cdtB* and *cdtC* genes identified in the present study with the 12 UPTC isolates appear to form an operon and to be functional in the cell, as with the six UN *C. lari* isolates shown previously.[14]

In addition, the putative ORFs for *cdtA* from the 12 UPTC isolates showed 74.2–100% nucleotide sequence similarities to each other. The figures for *cdtB* and *cdtC* were 78.2–100% and 68.4–100%, respectively (data not shown). The putative ORFs for *cdtA*, *cdtB* and *cdtC* from the 12 isolates showed 75.7–83.9%, 78.0–90.9% and 68.0–86.7% nucleotide sequence similarities to those of the six UN *C. lari* isolates, respectively (data not shown).

In relation to the adjacent genetic loci of the *cdt* gene operon analysed, a partial and putative ORF (np 1–329 bp) of the peptidase T (*pepT*) gene (Fig. 1) and two putative promoter structures (–35 region [AATCAA; np 392–399 bp]

A

UPTC Isolate	Primer employed (extension time)
CF89-14, A1	UPTCcdtallF / UPTCcdtall R2 / (1.5 m)
A2 and 92251	
NCTC12892	f-Clcdtall / cdtprobeR (45 s)
and 12893	cdtB_I428F / lpxB_196R (45 s)
A3	Clcdt_F4 / cdt1502R (45 s)
	cdtB_334F / Clcdt_R3 (45 s)
89049	UPTCcdtallF /1502R (45 s)
	cdtB_334F / Clcdt_R3 (45 s)
NCTC12894	UPTCcdtallF / cdt1502R (45 s)
and 1289	cdtB_I428F / cdtC567R (20 s)
	cdt1854F / 128946_cdtdown350R (10 s)
	128946_cdtCF / 128946_cdtCR (45 s, I-PCR)
NCTC12895	UPTCcdtallF / cdt1502R (45 s)
	cdtB_334F / lpxB_57R (30 s)

B

Primer	Primer sequence (5'-3')
UPTCcdtallF	AAAGAGCTGTGGTGATGGTG
UPTCcdtalR2	GTAAAGGTTTTTCCTGTGCC
f-Clcdtall	GGATGGCATGATACCTTTAGCTCC
cdtprobeR	AAATCTCCCATAATCATCC
cdtB_I428F	TAACGGCAGTGGATATGC
lpxB_196R	YYTCYAYAAAACCCATAGC
Clcdt_F4	CTTATATCTTTTAAGCCTAG
cdtB_334F	GCTTCAAGACCTATTATAGG
cdt1502R	TTTCCKGTAATTGCATAATC
cdtC567R	TGGAGTTGCTTCAACGATAGCAG
cdt1854F	KMAACKTCCTTTTGGTTATG
128946_cdtdown350R	TGTTTCGTTTAAATAGGTGC
128946_cdtCF	TATCGTTGAAGCAACTCC
128946_cdtCR	CTTCAACGATAGCAGGAG
lpxB_57R	TTCTTTTAAATGCAAAKTTG
Clcdt_R3	TCATGTGAGCTATAAAGTGG

Fig. 2. (A) Several kinds of PCR primer pairs designed *in silico* for the amplification of the *cdt* gene operons and their adjacent genetic loci from the 11 UPTC isolates. m, minutes; s, seconds. (B) Sequences of the primers.

Table 2. Putative ORFs of *cdtA*, *cdtB* and *cdtC* from *C. lari* isolates and *C. jejuni* reference strains.

Campylobacter		*cdtA*		*cdtB*		*cdtC*	
		ORF	CMW (Da)	ORF	CMW (Da)	ORF	CMW (Da)
UPTC	CF89-12	804	30,110	804	29,258	576	21,485
UPTC	CF89-12	804	30,110	804	29,258	576	21,485
UPTC	NCTC12892	798	30,126	804	29,458	573	21,354
UPTC	NCTC12893	798	30,174	804	29,503	573	21,408
UPTC	NCTC12894	792	29,803	804	29,174	567	20,936
UPTC	NCTC12895	792	29,793	804	29,201	567	20,866
UPTC	NCTC12896	792	29,803	804	29,174	567	20,936
UPTC	A1	804	30,182	804	29,239	570	21,611
UPTC	A2	804	30,166	804	29,239	570	21,611
UPTC	A3	804	30,194	804	29,239	570	21,715
UPTC	89049	804	30,166	804	29,241	570	21,702
UPTC	92251	804	30,180	804	29,239	570	21,611
UN *C. lari*	JCM2530T	810	30,351	804	29,333	573	21,886
UN *C. lari*	264	810	30,391	804	29,333	573	21,886
UN *C. lari*	298	810	30,391	804	29,333	573	21,886
UN *C. lari*	84C-1	810	30,391	804	29,332	573	21,886
UN *C. lari*	84C-2	810	30,391	804	29,332	573	21,886
UN *C. lari*	448	810	30,351	804	29,333	573	21,886
UN *C. lari*	RM2100	810	30,381	777	28,213	570	21,886
C. jejuni	RM1221	804	29,946	795	28,942	567	21,157
C. jejuni	NCTC11186	804	29,918	795	28,972	567	21,157

ORF: open reading frame; CMW: calculated molecular weights; Da: dalton.

and –10 region [ATTATA; np 359–364 bp] and an RB site [TTTCCT; np 334–339 bp) were identified to occur in the reverse direction immediately upstream of the *cdt* gene operon with the UPTC CF89-12. The partial and putative ORF of the *pepT* gene was also identified in the other 11 UPTC isolates (Fig. 5). In addition, some other complete or partial ORFs, type III restriction enzyme R protein (*res*; np 2769–5609 bp), adenine-specific DNA methyltransferase (*mod*; np 5605–7443 bp), hypothetical protein Hpu1 M9_07576 (np 7456–8505 bp) and partial and putative lipid-A-disaccharide synthase (*lpxB*) gene (np 9023–9418 bp) were also identified to occur in an approximately 6.6 kbp sequence downstream of the *cdt* gene operon in the UPTC CF89-12 isolate (Fig. 1).

Regarding the other 11 UPTC isolates, partial and putative *res* gene in UPTC CF89-14, A1, A2 and 9225, complete major antigenic peptide PEB3 and partial and putative *lpxB* in NCTC12892 and 12893, complete hypothetical protein Hpu1 M_907561, partial hypothetical protein Hpu1 M9_07566 and partial and putative *lpxB* in A3 and 89049, and putative *mod* in NCTC12894 and 12896, and partial and putative *mod, res* and *IpxB* in NCTC12895 were identified to occur downstream of each *cdtC* gene. Thus, the heterogeneous order and combination of the insertion genes appear to occur downstream of the *cdt* gene operon among the UPTC organisms examined.

Regarding the region downstream of the *cdt* gene operon in the UPTC organisms examined in the present study, an attempt was made to amplify the regions by using the newly constructed primer pair, 128946_*cdt*CF and *lpxB*_196R. As variable-length amplicons were generated with all 12 UPTC isolates using the primer pair (Fig. 6), the insertion genes with heterogeneous order and combination were also suggested to occur within the regions, being different from those of UN *C. lari*, as shown in previous work.[14]

Discussion

This is the first description of molecular and comparative analyses of the full-length *cdt* gene operon and its adjacent genetic loci from the UPTC organisms, and their comparison with those from other campylobacters.

Regarding the putative ORF for the *cdtB* gene (804 bp in length), this research group has already cloned, sequenced and analysed the *cdtB* gene fragments of approximately 720 bp in length from 24 isolates (n=16 UN *C. lari*, n=8 UPTC), obtained from different sources and in various countries.[13] Then, genetic heterogeneity of the *cdtB* gene locus among the isolates of *C. lari* was demonstrated. The nucleotide sequences of the partial *cdtB* gene fragments of 16 UN *C. lari* isolates showed 81–100% similarity. With eight UPTC isolates (UPTC CF89-12, CF89-14, NCTC12892, NCTC12893, A1, A3, 89049 and 92251) examined in the previous study,[13] as well as four new UPTC isolates (UPTC NCTC12894, 12895, 12896 and A2) in the present study, the identical polymorphic sites also occurred within the approximately 720 bp segment in the present study. Thus, the genetic heterogeneity of the *cdtB* gene locus among the 28 *C. lari*

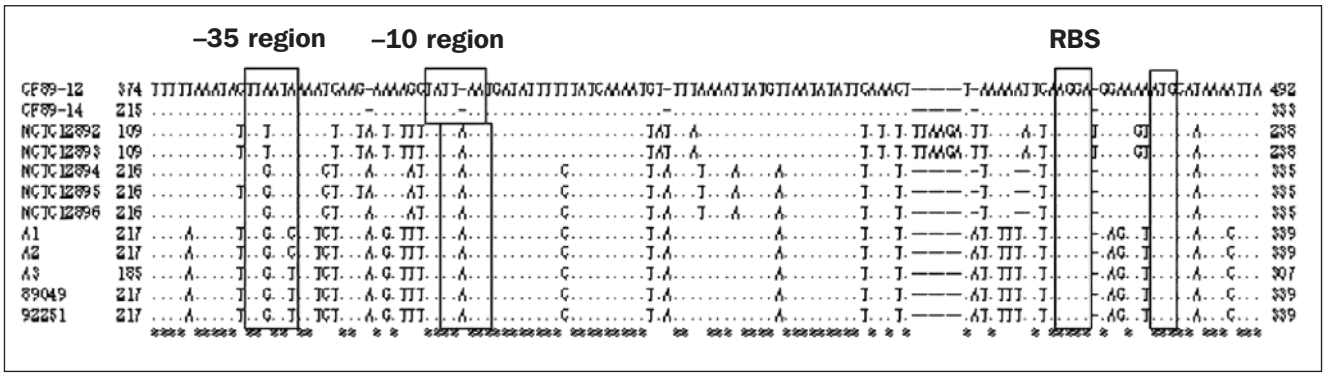


Fig. 3. Nucleotide sequence alignment analysis of an approximately 120 bp non-coding region including the typical promoter structures upstream of the start codon for the *cdtA* gene from the 12 UPTC isolates. Dots indicate identical bases; changes are indicated; dashes are deletions; numbers at the left and right refer to base pairs of the *cdt* gene operon sequences of the isolates examined in the present study. Positions identical in all the isolates are marked by asterisks.

isolates (n=16 UN *C. lari*, n=12 UPTC) was also demonstrated, respectively.

In the present study, deduced amino acid sequence alignment analyses were also carried out for putative ORFs of the full-length *cdtA, cdtB* and *cdtC* genes of UPTC isolates. The putative ORFs from the 12 UPTC isolates showed 52.1–100%, 66.4–100% and 50.8–100% deduced amino acid sequence similarities, respectively. They also showed 52.1–60.9%, 66.4–68.7% and 50.8–67.2% amino acid sequence similarities with those of *C. jejuni* RM1221 and NCTC11186 strains, respectively. Regarding the sequence similarity of the *cdt* genes, it is interesting that the nucleotide and deduced amino acid sequence of *cdtA, cdtB* and *cdtC* gave 100% similarity to each other between UPTC NCTC12894 and NCTC12896 (data not shown), as well as the promoter and terminator regions (Figs. 3 and 4).

It has been reported that CdtB of *Escherichia coli* shows significant position-specific homology to type I mammalian DNases, as specific DNaseI residues are involved in enzyme catalysis, DNA binding and metal ion binding.[27] Previously, the authors showed that all nine amino acid residues specific for both *E. coli cdtB* ORF and mammalian DNaseI were completely conserved in the *cdtB* gene locus among the 25 *C. lari* isolates examined, including *C. lari* RM2100, *C. jejuni* RM1221 and *C. coli* RM2228 strains.[14] In the present study, the results were reconfirmed with the *C. lari* islates (n=17 UN *C. lari, n*=12 UPTC). However, in the *C. lari* organisms that the genetic heterogeneity of the *cdtB* gene locus occurred, all nine amino acid-specific DNaseI residues[27] were completely conserved in the *cdtB* gene locus in all the *C. lari* isolates examined.

As described previously,[14] no PCR amplicons obtained from the UPTC isolates using primer pair f-/r-Cl*cdt*all, which could successfully amplify the full-length *cdt* gene operon, including the putative promoter and terminator structures (approximately 2.5 kbp) with the six UN *C. lari* isolates, could be generated. When, in the present study, molecular anatomical analyses of the full-length *cdt* gene operon and its adjacent genetic loci were successfully carried out with the 12 UPTC isolates using the other PCR primers, the f-/r-Cl*cdt*all primers[14] were shown theoretically to be able to hybridise the corresponding regions in some UPTC isolates examined. For example, the primer pair f-/r-Cl*cdt*all showed 79% (forward primer) and 92% (reverse primer) nucleotide sequence similarity to the corresponding regions in UPTC CF89-12, and 79% and 88% for 89049 and 79% and 83% for A3. Consequently, the variable gene insertions with the heterogeneous order and combinations occurred between

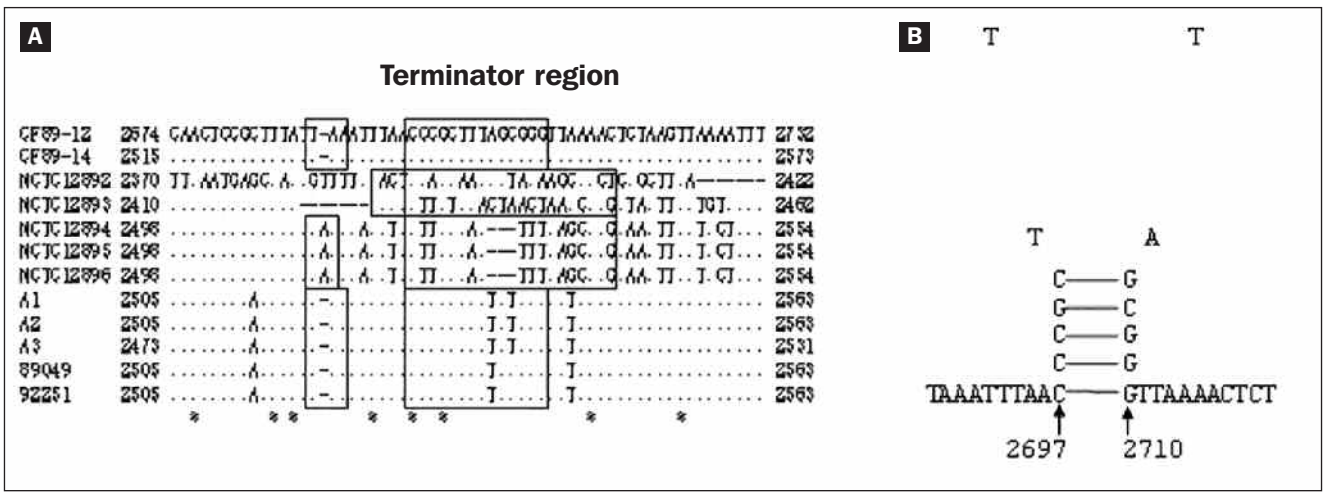


Fig. 4. Nucleotide sequence alignment analysis of the approximately 120 bp region downstream of the stop codon for the *cdtC* gene from the 12 UPTC isolates examined in the present study (A). For others in this Figure see the legend to Figure 3. (B) A case of putative intrinsic ρ-independent transcriptional terminator structure of UPTC CF89-12, which contains a G+C-rich region near the base of the stem.

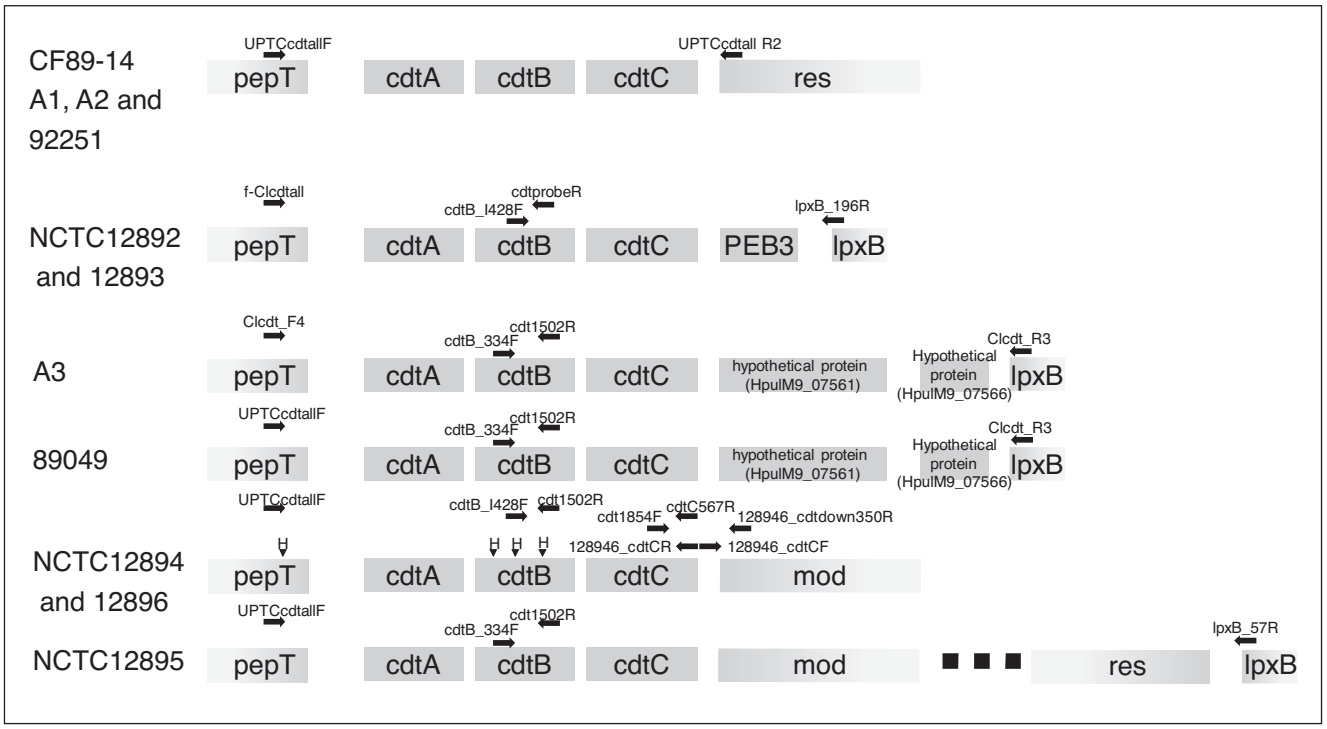


Fig. 5. Schematic representations of the *cdt* gene operons and their adjacent genetic loci from the UPTC isolates examined in the present study. Six kinds of the schematic representations are shown because of the occurrence of the identical loci.

the *cdtC* and *lpxB* genes, downstream of the *cdt* gene operon in the UPTC organisms examined. No PCR amplicons were generated with the UPTC isolates using the previous PCR conditions.[14]

Thus, this is a first molecular anatomical characterisation for the UPTC organisms to carry the full-length *cdt* gene operon, as well as two putative promoters and a hypothetically intrinsic ρ-independent transcription terminator within their genome DNA. In addition, variable gene insertions with heterogeneous order and combinations occurred characteristically between the *cdtC* and *lpxB* genes in the UPTC organisms. However, nucleotide sequencing of all the amplicons shown in Figure 6 could not be carried out because, as the authors have already described,[14] UN *C. lari* isolates carry no insertion genes between *cdtC* and *lpxB*, as is the case with the *C. lari* RM2100 strain. This is a characteristic difference between UN *C. lari* and UPTC.

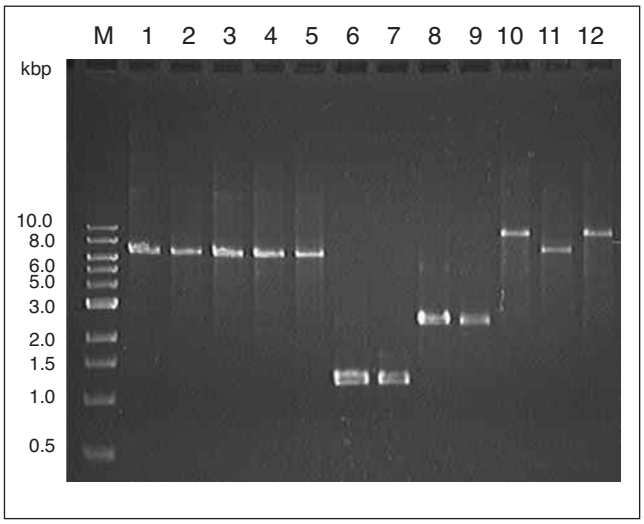


Fig. 6. Agarose gel electrophoresis profiles of PCR products generated with the 12 UPTC isolates using a primer pair of 128946_*cdt*CF and *lpxB* 196R to amplify the regions between the *cdtC* and *lpxB* genes. Lane M: 1kb DNA ladder; lane 1: UPTC CF89-12 isolate; lane 2: CF89-14; lane 3: NCTC12892; lane 4: 12893; lane 5: 12894; lane 6: 12895; lane 7: 12896; lane 8: A1; lane 9: A2; lane 10: A3; lane 11: 89049; lane 12: 92251.

As shown in the present study, UPTC organisms carry a complete *cdt* gene operon which conserves all the nine amino acid-specific DNaseI residues in their *cdtB* genes, and the urease gene operon,[28-30] which may be a virulent pathogen. However, whether or not the UPTC organisms are associated with gastrointestinal or other human disease remains unclear,[2] although four human UPTC isolates were obtained from patients in France in 1988 and 1990.[19,20] These may be partially due to the occurrence of short *flaA*-like sequences containing internal termination codons (TAG), incomplete or pseudogenes of *flaA*, and no flagellin or flagella in the UPTC organisms.[21,31–33] Therefore, it is especially interesting to clarify the functions of CDT, a representative virulence factor, in UPTC cells. □

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