Structural analysis and expression of the full-length cytochrome *P450* gene operon in *Campylobacter lari*

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ABSTRACT

Two sets of PCR primers are constructed to clone the cytochrome *P450* structural gene, including putative promoter and terminator structures, and its adjacent genetic loci in *Campylobacter lari* isolates. The putative open reading frames (ORFs) of the *P450* genes from 11 *C. lari* isolates (n=5 for urease-negative (UN) *C. lari*; n=6 urease-positive thermophilic campylobacters [UPTC]) examined consisted of 1365 or 1371 bases (455 or 457 amino acid residues), differing from those of the other thermophilic campylobacters (1359 [453] for *C. jejuni* and *C. upsaliensis*; 1368 [456] for *C. coli*). Each of the putative ORFs from the 11 isolates examined was also shown to carry start and stop codons and ribosome binding sites. Two putative promoter structures, consisting of sequences at the –35- and –10-like regions were also identified upstream of the ORFs. A single copy of the *P450* gene in the genome was identified with UN *C. lari* JCM2530^T and UPTC CF89-12, based on Southern blot hybridisation analysis. In addition, when reverse transcription polymerase chain reaction (RT-PCR) analyses were carried out, the transcription of the *P450* structural gene in *C. lari* organisms *in vivo* was confirmed. The transcription initiation site for the gene was also determined. High nucleotide sequence similarities (95.2–98.8%) of the full-length *P450* structural gene were shown with each of the 12 *C. lari* isolates. The UN *C. lari* and UPTC organisms showed similar findings with the neighbour-joining method, based on the sequence information of the *P450* structural gene.

KEY WORDS: Amino acid sequence.
Base sequence.
Campylobacter lari.
Cytochrome P450.
Gene expression.

Introduction

Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are a recognised cause of acute bacterial enteritis worldwide. *C. lari* is a thermophilic *Campylobacter* species first isolated from mammalian and avian species, particularly seagulls of the genus *Larus*.[1,2] *C. lari* is also an infrequent cause of clinical infection.[3,4] In addition, an atypical group of urease-positive thermophilic campylobacters (UPTC) was isolated from the natural environment in England in 1985[5] and described as a biovar or variant of *C. lari*.[6,7] Thus, the two representative taxa, namely urease-negative (UN) *C. lari* and UPTC, occur within the *C. lari* species.[8]

The cytochrome P450 is a diverse group of heme-containing enzymes that catalyse a wide range of oxidative reactions in eukaryotes and prokaryotes.[9] Studies on bacterial P450s have contributed to much current understanding of P450 mechanism and structure. Regarding the *P450* genes, genome sequence analyses have revealed that the number of genes varies between different organisms.[9] For example, the pathogenic bacterium *Mycobacterium tuberculosis* contains 20 genes and the Gram-positive model bacterium *Bacillus subtilis* contains eight genes. In addition, the model Gram-negative bacterium *Escherichia coli* contains none.[9,10]

In relation to *P450* genes of the thermophilic campylobacters, *P450* gene homologues encoding cytochrome P450 family proteins, probable cytochrome P450 or putative cytochrome P450, have recently been identified in *C. jejuni* RM1221 (DDBJ/EMBL/GenBank Accession No. NC_003912), *C. jejuni* subsp. jejuni NCTC11168 (NC_002163), *C. coli* RM2228 (AAFL01000008), *C. upsaliensis* RM3195 (AAFJ01000005) and *C. lari* RM2100 (AAFK01000002) strains, following whole genome shotgun sequencing analyses.[11] However, no reports have yet appeared for *C. lari* organisms.

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The aim of the present study is first to clone, sequence and characterise the full-length *P450* gene operon(s), including the putative promoter and terminator structures, among isolates of *C. lari* comprising UN *C. lari* and UPTC. These are then compared, and with those of the other thermophilic campylobacters, in order to clarify *P450* gene operon status in *C. lari* organisms. It also aims to confirm the expression of the gene in *C. lari* cells.

Materials and methods

Bacterial isolates including UN *C. lari* (n=5; JCM2530^T, 48, 298, 448 and 84C-1) and UPTC (n=6; NCTC12895, NCTC12896, 92251, 89049, CF89-12 and A3) analysed in the

Table 1. Description of *Campylobacter lari* isolates and some other thermophilic *Campylobacter* reference strains used in the present study.

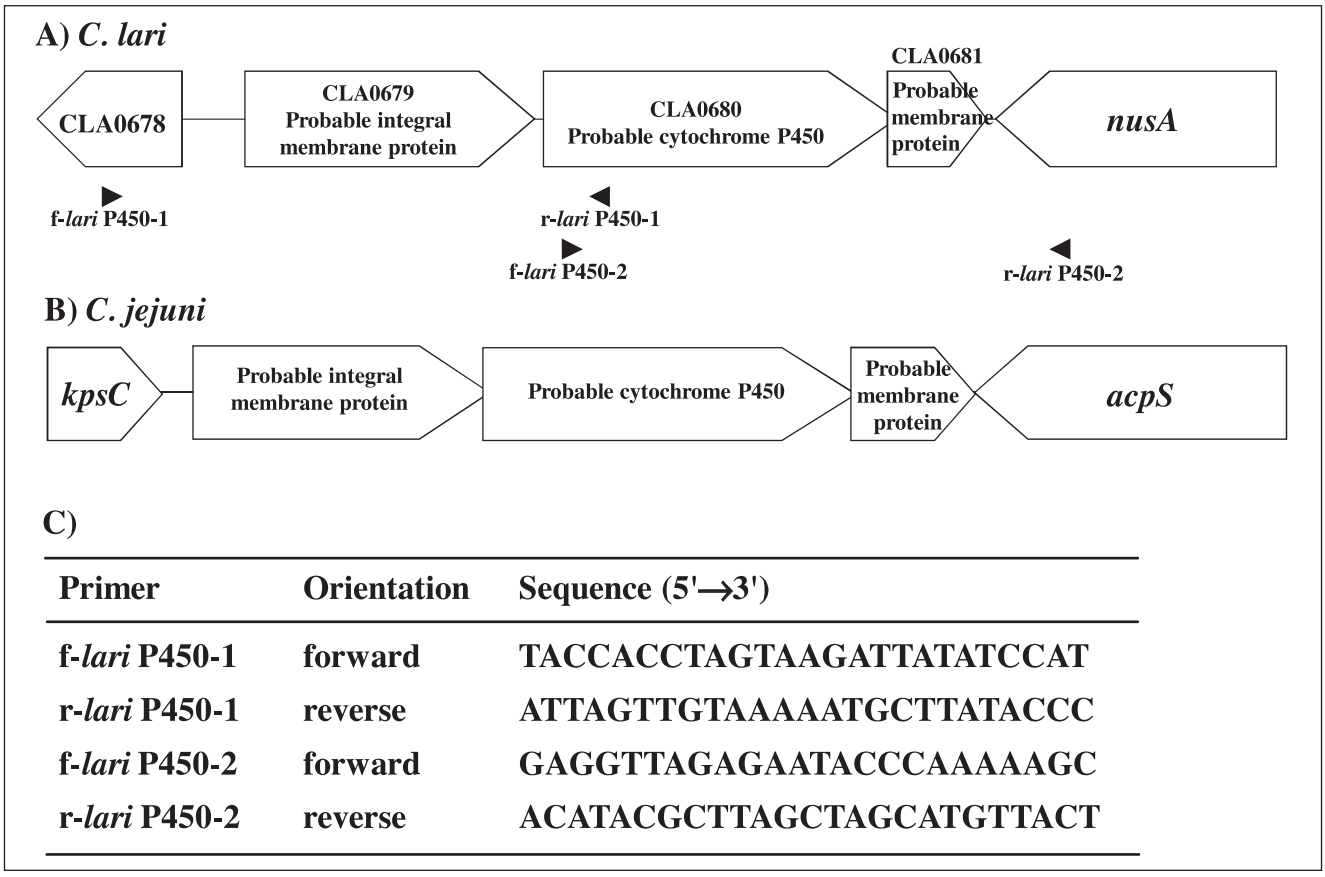
Isolate No.	Source	Country	P450 ORF	CMW (Da)	Accession No.
C. lari JCM2530T	Seagull	Japan	1,365	52,981	AB462021
C. lari 48	Food animal	Northern Ireland	1,371	53,196	AB462025
C. lari 298	Human	Japan	1,365	52,995	AB462023
C. lari 448	Mussels	Northern Ireland	1,371	53,195	AB464022
C. lari 84C-1	Human	Northern Ireland	1,371	53,308	AB462024
UPTC NCTC12895	Mussels	England	1,371	53,234	AB462016
UPTC NCTC12896	Mussels	England	1,371	53,205	AB462015
UPTC 92251	Human	France	1,371	53,210	AB462019
UPTC 89049	Human	France	1,371	53,251	AB462020
UPTC CF89-12	River water	Japan	1,371	53,172	AB462017
UPTC A3	Seagull	Northern Ireland	1,371	53,208	AB462018
C. lari RM2100	Human	USA	1,371	53,255	AAFK01000002
C. jejuni 260.94	Human	South Africa	1,359	52,705	AANK01000004
C. jejuni 81-176	Human	USA	1,359	52,644	NC_008787
C. jejuni 84-25	Human	Unknown	1,359	52,607	AANT02000001
C. jejuni CF93-6	Human	Japan	1,359	52,705	AANJ01000005
C. jejuni HB93-13	Human	China	1,359	52,632	AANQ01000001
C. jejuni NCTC11168	Human	UK	1,359	52,607	NC_002163
C. jejuni RM1221	Chicken	USA	1,359	52,607	NC_003912
C. coli RM2228	Chicken	Unknown	1,368	53,003	AAFL01000008
C. upsaliensis RM3195	Human	Unknown	1,359	53,100	AAFJ01000005

ORF: open reading frame; CMW: calculated molecular weight

present study are shown in Table 1. These isolates were cultured in Mueller-Hinton broth at 37°C for 48 h in an aerobic jar and on blood agar (Base No. 2, Oxoid, Hampshire, England) containing 7% (v/v) defibrinated horse blood (Nippon Bio-Test, Tokyo, Japan) and on *Campylobacter* selective medium (Virion, Zurich, Switzerland). 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). Genomic DNA was prepared from the cells using sodium dodecyl sulphate (SDS), proteinase K and cetyltrimethylammonium bromide treatment, and phenol-chloroform extraction and ethanol precipitation.[12]

	Campylobacter	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*C. lari* JCM2530^T	-	96.9	97.5	96.9	97.9	96.7	97.6	97.7	98.4	95.2	98.1	97.4	74.0	74.0	73.8	73.9	74.3	73.8	73.8	72.7	72.6
2	*C. lari* 48	98.5	-	96.9	98.7	97.0	96.5	96.8	96.8	97.5	95.5	97.4	97.2	73.7	73.6	73.6	73.7	74.2	73.6	73.6	73.0	71.9
3	*C. lari* 298	99.3	98.5	-	97.0	97.8	96.7	96.8	97.7	98.0	95.6	97.9	97.3	74.1	74.1	73.9	74.0	74.5	73.9	73.9	73.0	72.5
4	*C. lari* 448	98.7	99.8	98.7	-	97.0	96.5	96.8	96.8	97.7	95.5	97.7	97.2	73.7	73.6	73.6	73.7	74.2	73.6	73.6	73.0	71.9
5	*C. lari* 84C-1	98.7	98.0	98.5	98.3	-	97.1	97.8	98.2	98.6	96.0	98.6	97.9	74.0	74.0	73.8	73.9	74.3	73.8	73.8	73.3	72.6
6	UPTC NCTC12895	98.3	98.0	98.5	98.3	98.3	-	96.9	96.6	97.5	96.0	97.3	96.7	73.7	73.4	73.4	73.7	74.2	73.4	73.4	73.3	72.5
7	UPTC NCTC12896	98.9	98.7	98.7	98.9	98.5	98.5	-	98.0	97.8	95.7	98.2	96.9	73.7	73.7	73.7	73.6	74.2	73.7	73.7	72.9	72.5
8	UPTC 92251	98.9	98.7	98.7	98.9	98.5	98.5	99.6	-	98.3	95.7	98.5	97.5	73.9	73.9	73.7	73.8	74.4	73.7	73.7	72.9	72.6
9	UPTC 89049	99.3	98.7	98.7	98.9	98.9	98.5	99.1	99.1	-	95.6	98.8	98.0	74.3	74.0	73.8	74.2	74.6	73.8	73.8	73.8	72.7
10	UPTC CF89-12	97.6	97.8	97.8	97.6	97.6	98.5	97.8	97.8	97.8	-	96.1	95.8	72.9	72.9	72.8	72.8	73.4	72.8	72.8	72.1	72.3
11	UPTC A3	99.1	98.9	98.9	99.1	98.7	98.7	99.3	99.3	99.3	98.0	-	98.0	73.7	73.7	73.6	73.7	74.2	73.6	73.6	73.3	72.4
12	*C. lari* RM2100	98.3	98.5	98.5	98.7	98.7	98.3	98.5	98.5	98.5	97.6	98.7	-	73.7	73.4	73.4	73.6	74.2	73.4	73.4	72.9	72.7
13	*C. jejuni* 260.94	76.6	76.6	77.2	76.8	76.6	76.8	76.4	76.4	76.8	75.9	76.6	76.4	-	98.2	97.7	99.9	97.9	97.7	97.7	84.4	76.5
14	*C. jejuni* 81-176	76.2	76.2	76.8	76.4	76.2	76.4	76.9	75.9	76.4	75.9	76.2	75.9	99.1	-	97.9	98.3	97.4	97.9	97.9	84.4	76.1
15	*C. jejuni* 84-25	76.6	76.6	77.0	76.8	76.4	76.6	76.4	76.4	76.8	76.2	76.6	76.2	98.9	98.9	-	97.7	98.0	100.0	99.9	84.4	76.5
16	*C. jejuni* CF93-6	76.6	76.6	77.2	76.8	76.6	76.8	76.4	76.4	76.8	75.9	76.6	76.4	100.0	99.1	98.9	-	97.8	97.7	97.8	84.3	76.5
17	*C. jejuni* HB93-13	76.8	76.8	77.2	77.0	76.6	76.8	76.6	76.6	77.0	75.9	76.8	76.4	98.9	98.5	99.1	98.9	-	98.0	98.1	84.7	76.7
18	*C. jejuni* NCTC11168	76.6	76.6	77.0	76.8	76.4	76.6	76.4	76.4	76.8	76.2	76.6	76.2	98.9	98.9	100.0	98.9	99.1	-	99.9	84.4	76.5
19	*C. jejuni* RM1221	76.6	76.6	77.0	76.8	73.4	76.6	76.4	76.4	76.8	76.2	76.6	76.2	98.9	98.9	100.0	98.9	99.1	100.0	-	84.3	76.5
20	*C. coli* RM2228	75.6	75.8	76.0	76.0	75.6	75.8	75.6	75.6	75.8	75.3	75.6	75.3	91.7	91.2	91.2	91.7	91.7	91.2	91.2	-	76.8
21	*C. upsaliensis* RM3195	74.8	74.0	75.4	74.2	74.7	74.7	74.5	74.5	74.7	73.8	74.5	74.2	83.7	84.0	83.7	83.7	83.7	83.7	83.7	82.5	-

Table 2. Sequence similarities (%) of the nucleotide (upper right) and deduced amino acid (lower left) of the full-length *P450* structural gene from 12 *C. lari* isolates and other thermophilic *Campylobacter* reference strains.



C)

Primer	Orientation	Sequence (5'→3')
f-*lari* P450-1	forward	TACCACCTAGTAAGATTATATCCAT
r-*lari* P450-1	reverse	ATTAGTTGTAAAAATGCTTATACCC
f-*lari* P450-2	forward	GAGGTTAGAGAATACCCAAAAAGC
r-*lari* P450-2	reverse	ACATACGCTTAGCTAGCATGTTACT

Fig. 1. A schematic representation of the full-length cytochrome *P450* gene operon arrangement and its adjacent genetic loci in *C. lari* RM2100 and *C. lari* isolates, including the location of two primer pairs (A) for PCR amplification and primer details (C). The gene arrangement for *C. jejuni* RM1221 strain is also shown for comparison (B).

Two PCR primer pairs (f-/r-*lari*P450-1 and f-/r-*lari*P450-2) were prepared *in silico* for amplification of the full-length *P450* gene, including the putative promoter and terminator structures and adjacent genetic loci of *C. lari* isolates, based on the sequence information of the *P450* structural gene of *C. lari* RM2100 (AAFK01000002),[11] as shown in Figure 1 (*C. jejuni* RM1221 gene arrangement also shown for comparison [B]).

The PCR mixture contained 100-ng template DNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 100 µmol/L each dNTP, 0.6 µmol/L each primer, and one unit EX *Thermus aquaticus* (*Taq*) DNA polymerase (Takara Bio, Shiga, Japan). The PCR was performed in a 50 µL volume at 94°C for 3 min, for 30 cycles at 94°C for 1 min, 48°C (or 52°C) for 30 sec, 72°C for 1 min 40 sec, and finally 72°C for 7 min.

Amplified PCR products were separated by 1% (w/v) agarose gel electrophoresis in 0.5xTBE, then extracted and purified from the gel, as described previously.[13,14] Purified PCR products were cloned into pGEM-T vector (Promega, Tokyo, Japan) using the TA cloning procedure. Following nucleotide sequencing with M13, sequencing of the amplicons was performed (Hitachi SQ5500EL DNA autosequencer). Sequence analysis was carried out using

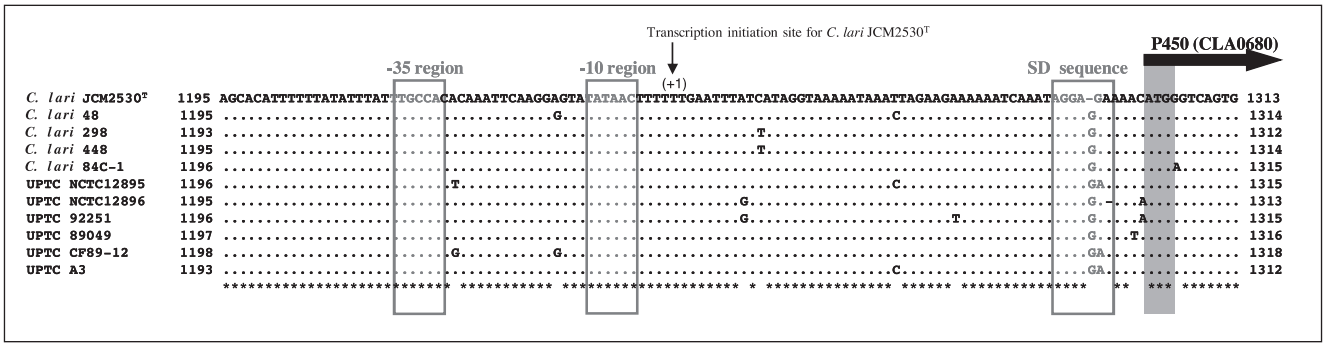


Fig. 2. Nucleotide sequence alignment analyses of approximately 120 bp NC region, including typical promoter structures of the 11 *C. lari* isolates examined, including *C. lari* RM2100. Dots indicate identical bases; changes are so indicated; dashes are deletions; positions identical in all isolates are marked by asterisks; numbers at the left and right refer to the nucleotide position in the each isolate.

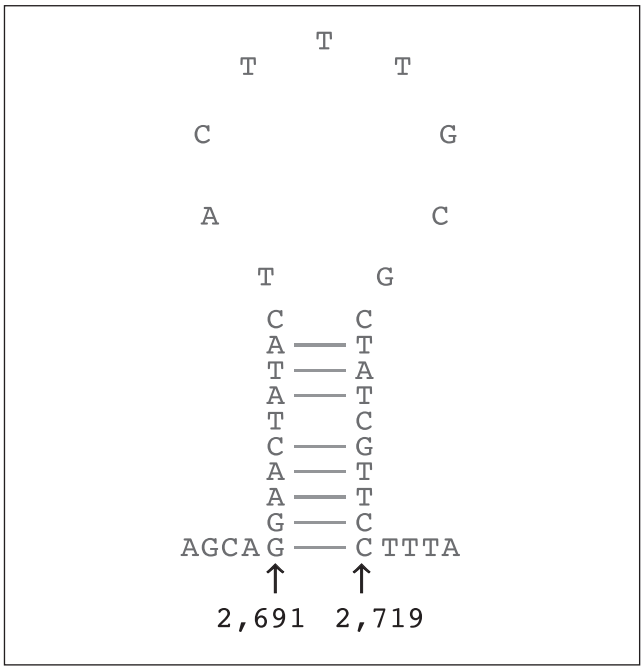


Fig. 3. A putative intrinsic ρ-independent transcriptional terminator structure which contains a G+C-rich region near the base of the stem and a single-stranded run of T residues.

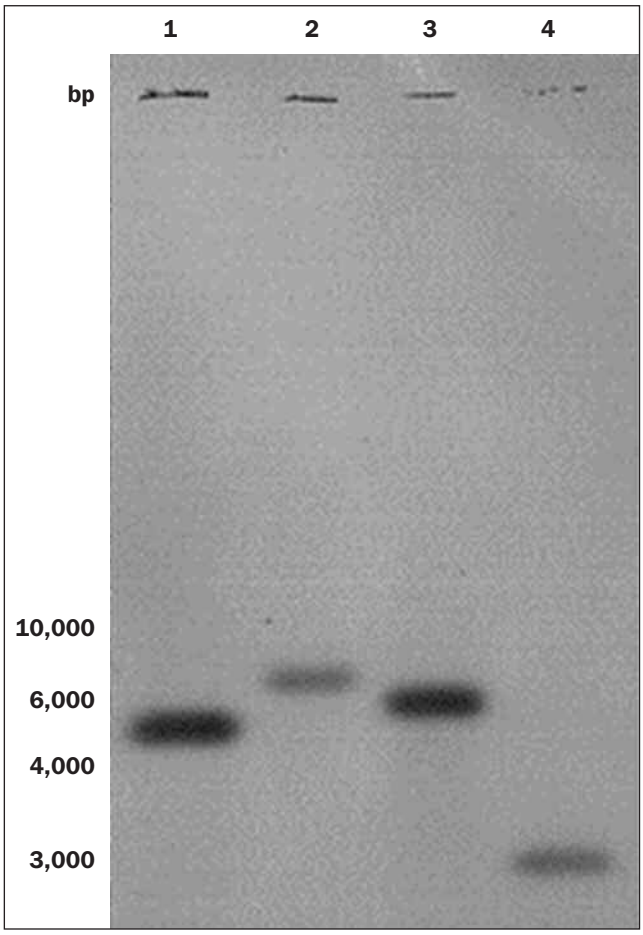


Fig. 4. Southern blot hybridisation analysis of genomic DNA separately digested with *Hha* I (lanes 1 and 2) and *Psh* B I (lanes 3 and 4) from *C. lari* JCM2530^T (lanes 1 and 3) and UPTC CF89-12 (lanes 2 and 4) isolates, using a nearly full length P450 structural gene fragment from *C. lari* JCM2530^T as the probe.

the Genetyx-Mac (version 9; Genetyx, Tokyo, Japan) computer software. In order to achieve accuracy, multiple TA-cloned PCR products were sequenced. Moreover, the sequences corresponding to the PCR primers of f-*lari*P450-1 and r-*lari*P450-2 were excluded from the sequence data of the approximately 3.1-kbp region containing the full-length *P450* gene operon, which is now accessible in the DDBJ/EMBL/GenBank (Table 1). The nucleotide positions used in the present study were those of *C. lari* JCM2530^T.

Southern blot hybridisation analysis of the *P450* gene was carried out using a digoxigenin (DIG)-labelled nearly full-length *C. lari P450* structural gene fragment (approximately 1250 bp) prepared from *C. lari* JCM2530^T as a probe with Hha I- and PshB I-digested whole-genome DNAs, according to the procedure described by Sambrook and Russell.[12] The fragment probe was amplified using a degenerate primer pair (f-/r-*lari*P450-3; f-*lari*P450-3, 5′-CTTGATGGRCTTTATGAGCGAAG-3′, nucleotide positions [np] 1387–1409 bp for *C. lari* JCM2530^T and r-*lari*P450-3, 5′-GCYGATCTTATAGTHARTCTTCC-3′, np 2614–2636 bp [AB462021]) designed *in silico* in the present study. Random primer extension was performed in order to prepare the fragment probe using DIG-High Prime (Roche Applied Science, Penzberg, Germany).

The RT-PCR method was carried out with the f-*lari* P450-2 and r-*lari* P450-1 primer pair using the Qiagen OneStep RT-PCR kit (Qiagen, Tokyo, Japan). This primer pair was expected to generate a RT-PCR product of the *P450* structural gene segment of approximately 100 bp. Total cellular RNA was extracted and purified from *C. lari* cells using RNAprotect bacteria reagent and the RNeasy mini kit (Qiagen).

The transcription initiation site for the *P450* gene was determined by primer extension from the purified total cellular RNA of *C. lari* JCM2530^T cells. The primer selected for this analysis was 5′-CGCTCATAAAGTCCATCAAGCC-3′, which corresponds to the reverse complementary sequence (np: 1385–1406 bp). The transcription initiation site was determined by primer extension with the sizes of DNA fragments generated by sequencing reactions.

Nucleotide sequences of approximately 3.1 kbp of the full-length *P450* gene operon from 12 *C. lari* isolates including the *C. lari* RM2100 strain were compared and to accessible sequence data of other thermophilic campylobacters by employing CLUSTAL W software (1.7 program)[15] incorporated in the DDBJ. Following this, a phylogenetic tree was constructed by the neighbour-joining (NJ) method.[16]

Results and discussion

Following construction of the two PCR primer pairs for amplification of the *P450* gene operon, including the putative promoter and terminator regions, two amplicons were generated (each of approximately 1.6 kbp) and TA-cloned and sequenced *P450* structural genes and adjacent

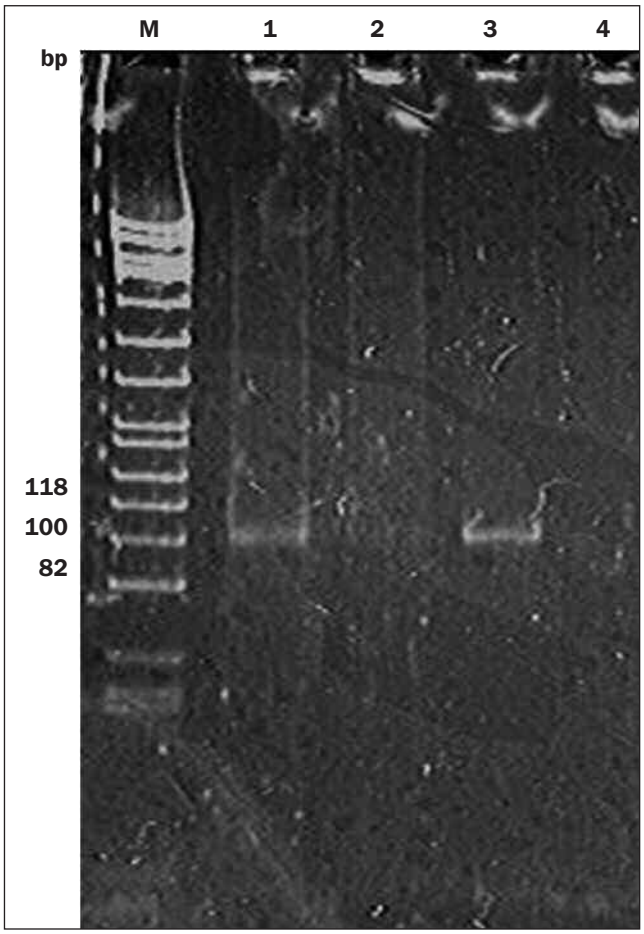


Fig. 5. RT-PCR analysis of the *P450* structural gene transcript expressed in the *C. lari* cells. Lane M: ϕx174 DNA *Hin*flmarkers (Promega, Tokyo, Japan); lanes 1 and 2: *C. lari* JCM2530^T; lanes 3 and 4: UPTC CF89-12; lanes 1 and 3: with the total cellular RNA and reverse transcriptase (RTase) XL; lanes 2 and 4: with the total cellular RNA but without RTase.

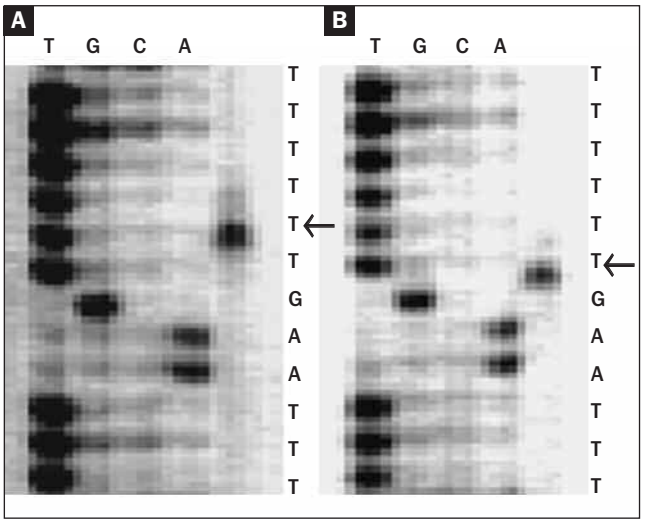


Fig. 6. Primer extension analysis from the purified total cellular RNA of *C. lari* JCM2530^T (A) and UPTC CF89-12 (B). The arrows indicate the transcription initiation sites.

genetic loci (approximately 3.1 kbp combined sequences) from all 11 *C. lari* isolates.

Based on sequence information of the *P450* structural gene from the *C. lari* RM2100 reference strain (EAL55246/AAFK01000002; probable cytochrome P450 Cj1411C), the putative open reading frames (ORFs) of *P450* from the 11 isolates examined were shown to have 1365 or 1371 bases (np 1303–2667 bp for *C. lari* JCM2530^T; 455 amino acid residues), differing from those of other thermophilic campylobacters (1359 [453 amino acid residues] for *C. jejuni* RM1211 and six other *C. jejuni* isolates, 1368 [456] for *C. coli* RM2228 and 1359 [453] for *C. upsaliensis* RM3195 [Table 1]). Thus, the sizes of the *P450* ORFs appear to be similar but not identical among the thermophilic *Campylobacter* isolates.

Two putative promoter structures, consisting of consensus sequences at the –35-like (TTGCCA; np 1215–1220 bp) and –10-like (TATAAC; np 1238–1243 bp) regions were identified as a transcriptional promoter, immediately upstream of the *P450* structural gene in all 11 *C. lari* isolates (Fig. 2).

Each of the putative *P450* ORFs from the 12 *C. lari* isolates including the RM2100 strain was shown to carry an ATG (np 1303–1305 bp) as the start codon and a TAA (np 2668–2670 bp) as the stop codon. Probable ribosome binding (RB) sites (Shine-Dalgarno [SD] sequences)[17] that are complementary to a highly conserved sequence of CCUCCU close to the 3′ end of 16 ribosomal RNA, AGGAG (G; np 1293–1297 bp) were also identified in the *P450* structural gene with all 12 isolates (Fig. 2). The putative ORF for the cytochrome P450 from the *C. lari* isolates was predicted with a calculated molecular weight (CMW) of approximately 53 kDa (Table 1).

Regarding the transcriptional terminator for the *P450* gene, a hypothetically intrinsic ρ-independent transcriptional terminator structure was similarly identified in the *P450* gene operon (between np 2691 and 2719 bp) in the 12 *C. lari* isolates (Fig. 3). It contained a G+C-rich region near the base of the stem and a single-stranded run of T residues (Fig. 3).

Regarding the regions immediately upstream and downstream of the putative *P450* ORFs within the combined sequences of approximately 3.1 kbp, two putative and full-length ORFs of the probable integral membrane protein (CLA0679; np 164–1291 bp) and the probable membrane protein (CLA0681; np 2673–3023 bp) were also identified in the 12 *C. lari* isolates (Table 1). The probable integral membrane protein (CLA0679) and the probable membrane protein (CLA0681) were identified to be 1131 bp and 354 bp for their structural genes in all *C. lari* isolates, respectively. In addition, the protein (CLA0679) was identified to be 1137 bp in *C. lari* 84C-1, UPTC92251 and UPTC CF89-12.

Two putative typical promoter structures, consisting of sequences at the –35 (TTGACA; np 102–107 bp) and –10 (TATATT; np 125–131 bp) regions, as well as the start codon (ATG; np 164–166 bp) for the probable internal membrane protein (CLA1679) gene, were identified for the transcriptional promoter in the 12 *C. lari* isolates (data not shown). For the probable membrane protein (CLA0681) gene, two putative promoter structures (CTGATA, np 2603–2608 bp; TATAAG, np 2625–2630 bp) were also identified in all the *C. lari* isolates examined.

Hypothetically intrinsic ρ-independent transcription terminator structures were also identified for the putative CLA0679 and CLA0681 genes (between np 1305 and 1321 bp

for CLA0679; between np 3042 and 3061 bp for CLA0681) (data not shown).

As shown in Table 2, the nucleotide sequences of the full-length *P450* genes showed 95.2–98.8% similarity. In addition, those from the 12 *C. lari* isolates showed 71.9–74.6% similarity with those from the other nine thermophilic campylobacters (n=7 *C. jejuni*, n=1 *C. coli* and *C. upsaliensis*) (Table 2).

Moreover, deduced amino acid sequence alignment analysis was also carried out for putative ORFs of the full-length *P450* gene of *C. lari* isolates, as well as those of the other thermophilic campylobacters. The putative ORFs of the *P450* from the 12 *C. lari* isolates, including the *C. lari* RM2100 strain, showed 97.6–99.8% amino acid sequence similarity and 73.4–77.2% similarity with those of the other nine thermophilic campylobacters (Table 2).

The deduced sequences of amino (N) terminal 30 amino acid residues showed extremely high sequence similarity (86.7–100%) among three thermophilic campylobacters (*C. lari, C. jejuni* and *C. coli*) (data not shown). However, the deduced sequences of N terminal 30 amino acid residues from the 11 *C. lari* isolates showed extremely low sequence similarity (~20%) compared with those from other bacterial *P450* examples (data not shown).

When Southern blot hybridisation analysis was carried out to clarify how many copies of the *P450* gene occur in the genome of *C. lari* isolates (Fig. 4), the occurrence of a single copy was demonstrated in both genomes of UN *C. lari* JCM2530^T (lanes 1 and 3) and UPTC CF89-12 (lanes 2 and 4) examined. This observation of a single copy of the *P450* gene in the genome appears to be a characteristic of *C. lari* organisms.

When RT-PCR analysis was carried out for the RNA components extracted from the UN *C. lari* JCM2530^T and UPTC CF89-12 cells, a single product (approximately 100 bp) was recognised in the gel (Fig. 5). Thus, the transcription of the *P450* structural gene was confirmed in the cells.

The transcription initiation site for the *P450* gene operon was determined by the primer extension procedure (Fig. 6). The +1 transcription initiation sites for the *P450* gene are underlined in the following sequences; 5′-TTTTTTGAATTT-3′ for *C. lari* JCM2530^T and 5′-TTTTTTGAATTT-3′ for UPTC CF89-12 (Fig. 6). Thus, the transcription initiation sites were identified to slide by one base between the two isolates, probably due to a single base deletion immediately upstream of the ATG start codon for the gene in *C. lari* JCM 2530^T.

Recently, Liu *et al*. carried out a comprehensive analysis of the occurrence of pseudogenes in a diverse selection of 64 prokaryote genomes, and the cytochrome *P450* gene was 13th among the 20 top-ranking domain families in terms of pseudogenes.[18] However, in the present study, the *P450* gene, identified to be a single copy in the *C. lari* genome, was not a pseudogene but it was transcribed.

A phylogenetic tree constructed using the NJ method based on the nucleotide sequence similarity data of the *P450*

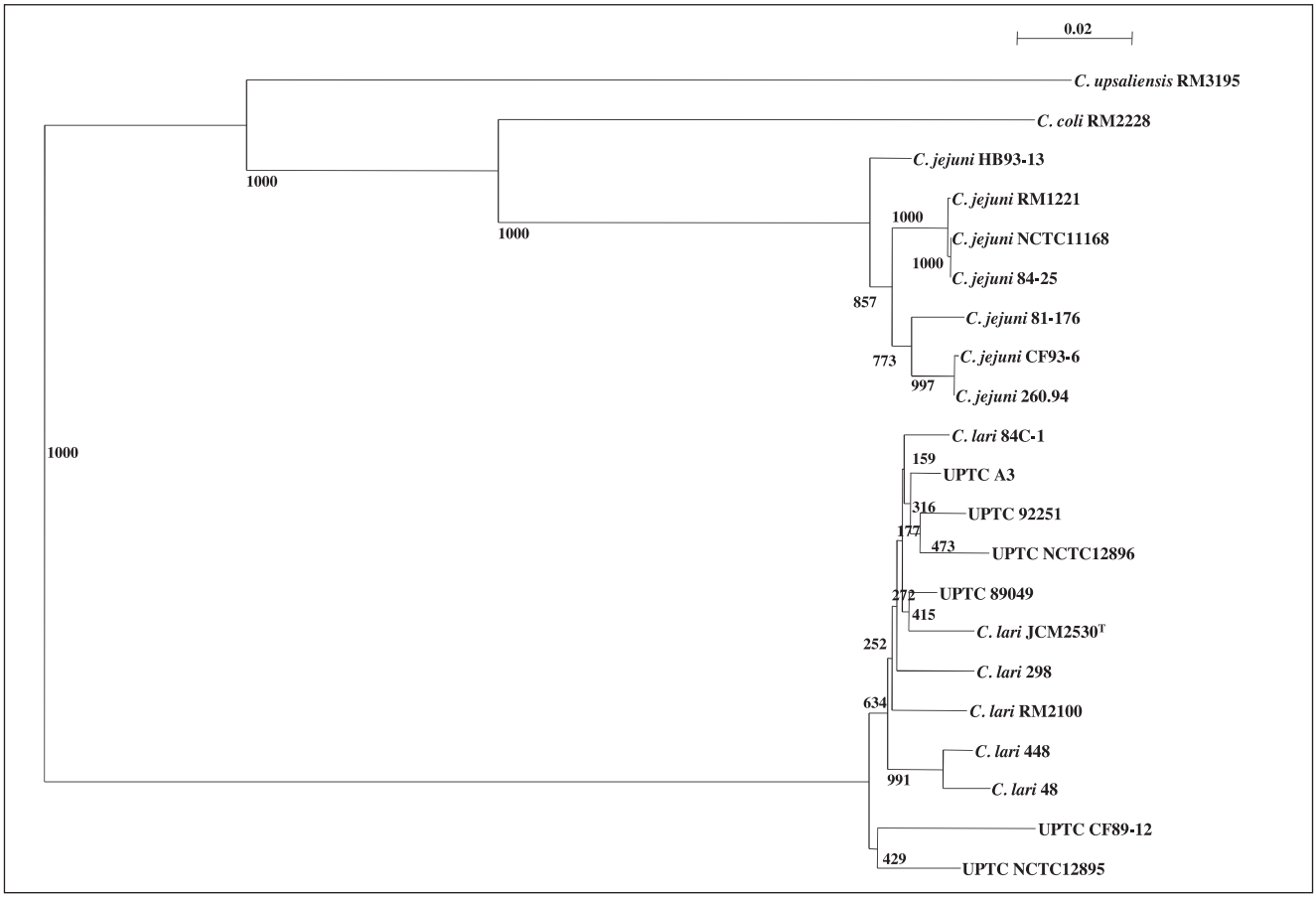


Fig. 7. A phylogenetic tree construction based on the nucleotide sequence information of the *P450* structural gene from 12 *C. lari* isolates and other thermophilic campylobacters. The tree was constructed by the NJ method using *C. upsaliensis* RM3195 as an out-group. Values (0.02) in the figure represent evolutionary distances. Boot-strap values of 1000 are shown at the branch point.

structural gene from the 12 *C. lari* isolates and other thermophilic *Campylobacter* reference strains accessible in the DDBJ/EMBL/GenBank is shown in Figure 7. There are some major clusters in the dendrogram constructed. The phylogenetic tree demonstrates that the 11 *C. lari* isolates and a reference UN *C. lari* RM2100 strain formed some minor clusters showing genetic hypervariability, separating these taxa from other thermophilic campylobacters (i.e., *C. jejuni, C. coli* and *C. upsaliensis*). In addition, Figure 7 also shows that the NJ dendrogram of UN *C. lari* and UPTC organisms was similar based on the nucleotide sequence data of the *P450* structural gene. □

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