

Molecular cloning and characterisation of the methionine sulphoxide reductase A (*msrA*) gene locus in *Campylobacter lari* organisms

T. NAKAJIMA*, K. MATSUBARA†, J. E. MOORE^{‡§}, T. MURAYAMA† and M. MATSUDA*

*Graduate School of Environmental Health Sciences, Azabu University, Sagamihara;

†Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa, Japan;

‡Department of Bacteriology, Northern Ireland Public Health Laboratory, Belfast City Hospital; and §University of Ulster, Coleraine, Northern Ireland, UK

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Introduction

Protein oxidation is one of the consequences facing all organisms encountering oxidative stress.¹ In addition, reactive oxygen species such as superoxide, hydrogen peroxide and hypochlorous acid oxidise either free amino acid residues or residues within proteins.¹ One of the most oxidation-sensitive amino acids is methionine.

The reductive repair of oxidised methionine residues is performed by methionine sulphoxide reductase (Msr). Recently, Sasindran *et al.* focused primarily on the relationship between Msr and virulence of bacterial pathogens, bacterial adherence, bacterial biofilm formation, bacterial motility, intracellular survival bacteria, *in vivo* survival of bacteria resistant to oxidants, secretion, up-regulation, and protein oxidation and virulence.² Msr (MsrA and B) is encoded by two genes, *msrA* and *msrB*,² and MsrA and MsrB are highly conserved enzymes in prokaryotes and eukaryotes, respectively, but they share no sequence or structural similarities.^{3,4}

Thermophilic *Campylobacter lari* organisms were first isolated from seagulls.^{5,6} *C. lari* has also been shown occasionally to be a cause of clinical infection.⁷⁻⁹ In addition, an atypical group of urease-positive thermophilic *Campylobacter* (UPTC) organisms has been isolated from the natural environment in England in 1985.¹⁰ Thereafter, these organisms were described as a biovar or variant of *C. lari*.^{11,12} Subsequent isolates were reported in Europe¹¹⁻¹⁷ and in Japan.^{18,19} Thus, at least, these two representative taxa, urease-negative (UN) *C. lari* and UPTC exist within the *C. lari* species.²⁰

Regarding the Msr of *Campylobacter*, Parkhill *et al.* first identified that *C. jejuni* NCTC11168 contains putative *msrA*

ABSTRACT

The methionine sulphoxide reductase A (*msrA*) gene and its adjacent genetic loci from urease-negative (UN) *Campylobacter lari* RM2100 and urease-positive thermophilic *Campylobacter* (UPTC) CF89-12 strains appear to be composed of a *msrA* structure gene (507 base pairs [bp]) and another five-gene cluster (approximately 6300 bp) in the same strand and direction. A primer pair (F1/R4-*msrA*) for polymerase chain reaction (PCR) amplification was designed to generate a product of approximately 900 bp of the *msrA* gene, including its adjacent genetic loci for the thermophilic *Campylobacter* organisms and generate an amplicon with 16 *C. lari* isolates ($n=4$ for UN *C. lari*; $n=12$ for UPTC). Following direct nucleotide sequencing, sequence analysis and nucleotide sequence alignment analysis, the putative full-length *msrA* gene from the 16 *C. lari* isolates showed high nucleotide sequence similarities (91.8–100%) to each other and relatively low similarity (69.3–71.8%) to three reference *C. jejuni* and *C. coli* strains. In addition, the *msrA* gene was transcribed in both the UPTC CF89-12 and NCTC12893 cells using reverse transcription PCR. An immunoreactively positive signal was identified in the UPTC CF89-12 and NCTC12893 cells with anti-UPTC MsrA synthetic peptide antibodies.

KEY WORDS: *Campylobacter*.
DNA sequencing.
Polymerase chain reaction.

and *msrB* genes with unknown roles, and the deduced MsrA is encoded by Cj0637c and MsrB by Cj1112c.²¹ In addition, Attack and Kelly described the contribution of the stereospecific methionine sulphoxide reductases, MsrA and MsrB, to be oxidative and nitrosative stress resistance in the foodborne pathogen *C. jejuni* NCTC11168 strain.²²

However, a description of the *msrA* in *C. lari* has yet to appear. Therefore, the aim of the present study is to clone, sequence and characterise the full-length *msrA* structural gene and its adjacent genetic loci from several *C. lari* organisms, UN *C. lari* and UPTC using several degenerate polymerase chain reaction (PCR) primer pairs designed *in silico*. In addition, it aims to examine the possible copy number(s) of the putative *msrA* gene in the cells and to molecularly compare the nucleotide sequences and the deduced amino acid sequences of the *msrA* from 16 *C. lari* isolates to those from reference *C. jejuni* and *C. coli* strains. It will also describe *msrA* gene sequence expression at the transcriptional and translational levels.

Correspondence to: Professor Motosu Matsuda

Laboratory of Molecular Biology, Graduate School of Environmental Health Sciences, Azabu University, Fuchinobe 1-17-71, Sagamihara Chuo-ku, Kanagawa 252-5201, Japan

Email: matsuda@azabu-u.ac.jp

Materials and methods

Bacterial isolates, culture conditions and genomic DNA preparation

C. lari isolates ($n=4$ for UN *C. lari*, $n=12$ for UPTC) employed in the present study are shown in Table 1. Genomic DNA was prepared according to the procedure described by Harrington *et al.*²³ The DNA concentration was adjusted to approximately 800 ng/ μ L.

Construction of the genomic DNA library of the UPTC CF89-12 strain

A genomic DNA library was constructed using NEBNext DNA sample preparation reagent (Set 1; New England BioLabs, Tokyo, Japan). Fragmented DNA was obtained using Covaris S-series apparatus (Covaris, USA) and was subsequently separated by agarose gel electrophoresis (300–500 base pairs [bp]). Cluster generation was carried out using the constructed DNA library as templates with Cluster Station and Cluster Generation Kit (Illumina, USA).

Nucleotide sequence determination and analysis

The nucleotide sequence (sequence reads 75 bp) was determined using Genome Analyzer Iix and Sequencing Kit (Illumina). *De novo* assembly of the paired-end reads (35 bp) was carried out using Edena (V2.1.1.) and Velvet (V0.7.11). Nucleotide sequence analysis of the full-length *msrA* and its adjacent genetic loci was carried out using the GENETYX-Windows computer software version 9 (GENETYX, Tokyo, Japan).

Primer design, PCR amplification and product separation and purification

A degenerate primer pair (F1-/R4-*msrA*; Fig. 1) employed for the PCR amplification of a segment including the full-length of the *msrA* gene was designed based on sequence information taken from DDBJ/EMBL/GenBank (*C. lari* RM2100, NC_012039; *C. jejuni* NCTC11168, NC_002163; *C. jejuni* 260.94, NZ_AANK01000003; *C. jejuni* RM1221, CP000025; *C. jejuni* subsp. *doylei* 269.97, NC_009707; *C. coli* RM2228, NZ_AAFL01000001). The PCR mixture contained 1x KOD-Plus-ver. 2 buffer, 1.5 mmol/L $MgSO_4$, 200 μ mol/L each dNTP, 0.3 μ mol/L each primer, and a total of one unit of KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan). The PCR was performed in 50- μ L reaction volumes using the

Table 1. Isolates of *C. lari* analysed in the present study and accession numbers of the nucleotide sequence data accessible in DDBJ/EMBL/GenBank.

No.	Isolate No.	Source	Country	Accession No.
1	UPTC NCTC12893	River water	England	AB693884
2	UPTC NCTC12894	Sea water	England	AB693885
3	UPTC NCTC12895	Mussel	England	AB693886
4	UPTC NCTC12896	Mussel	England	AB693887
5	UPTC CF89-12	River water	Japan	AB702689
6	UPTC CF89-14	River water	Japan	AB693889
7	UPTC A1	Seagull	N. Ireland	AB693890
8	UPTC A2	Seagull	N. Ireland	AB693891
9	UPTC A3	Seagull	N. Ireland	AB693892
10	UPTC 89049	Human	France	AB693893
11	UPTC 92251	Human	France	AB693894
12	UPTC 99	Sea water	N. Ireland	AB693895
13	UN <i>C. lari</i> JCM2530 [†]	Seagull	Japan	AB693896
14	UN <i>C. lari</i> 176	Black-tailed gull	Japan	AB693897
15	UN <i>C. lari</i> 300	Seagull	USA	AB693898
16	UN <i>C. lari</i> 84C-1	Human	N. Ireland	AB693899
17	UN <i>C. lari</i> RM2100	Human	USA	NC_012039

primer pair F1/R1-*msrA*, F1/R2-*msrA* or F3/R4-*msrA* at 94 °C for 5 min, then 30 cycles at 94 °C for 0.5 min, at 44.4 °C, 45 °C or 49 °C for 0.5 min, and at 68 °C for 1.5 min, followed by a final extension at 68 °C for 7 min.

Amplified PCR products were then separated by 1% (w/v) agarose gel electrophoresis in 0.5x TBE at 100 V and detected by staining with ethidium bromide. Separated PCR products were purified using a QIAEX II gel extraction kit (Qiagen, Tokyo, Japan).

Nucleotide sequencing and nucleotide and deduced amino acid sequence analyses

The purified fraction was subjected to cycle sequencing with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Tokyo, Japan) and with sequencing primers. The reaction products were separated and detected on an ABI PRISM 3100 genetic analyser (Applied Biosystems). Sequence analysis was also carried out using the GENETYX Windows software (version 9; GENETYX, Tokyo, Japan), as described above.

Table 2. Summaries of the promoter, RBS and start codon for the *msrA* gene and its adjacent genetic loci within UPTC CF89-12.

No.	Promoter at -35 like region		Promoter at -10 like region		RBS		Start codon	
	Sequence	np (bp)	Sequence	np (bp)	Sequence	np (bp)	Sequence	np (bp)
2	TTAATA	2216–2221	TAAAAT	2237–2242	AGGAAA	2253–2258	ATG	2263–2265
3	TTTAGA	2925–2930	TAAATT	2963–2968	AGGAGC	2984–2989	ATG	2997–2999
4	NA	NA	NA	NA	AGGTGA	3,245–3,250	ATG	3265–3267
5	NA	NA	TATAAA	4330–4335	NA	NA	ATG	4358–4360
6	NA	NA	TAGAAG	5289–5294	AGGCGA	5302–5307	ATG	5329–5331
7	TGGAGA	5625–5630	TTTAAA	5655–5660	ATGAGG	5666–5671	ATG	5684–5686

Nos. 2 to 7, equivalent to the order of the gene no. shown in the Figure 2. NA: not available.

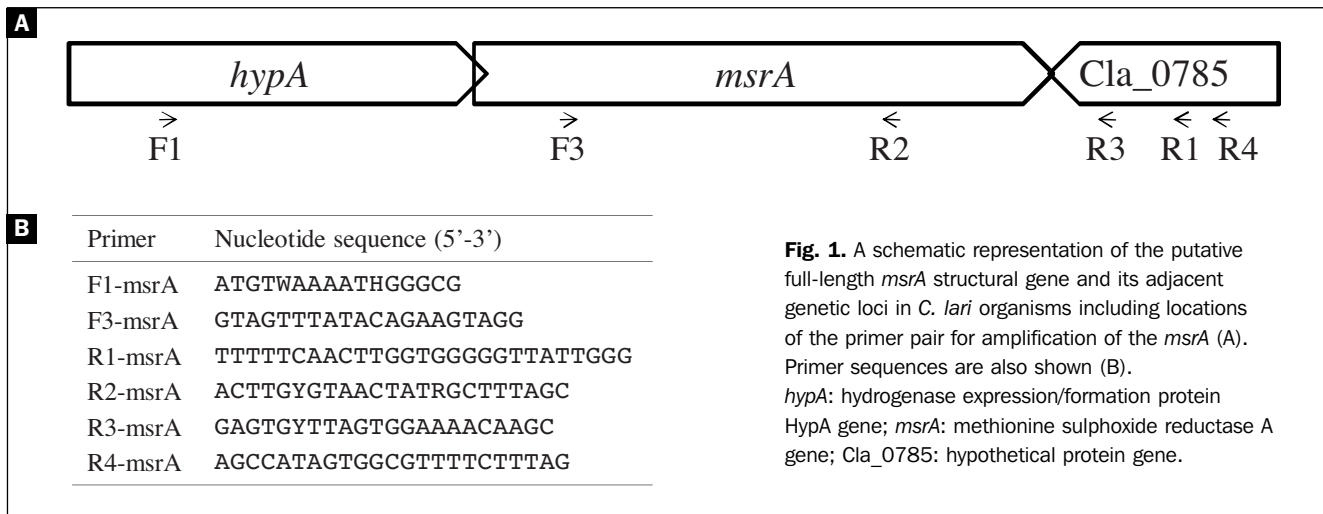


Fig. 1. A schematic representation of the putative full-length *msrA* structural gene and its adjacent genetic loci in *C. lari* organisms including locations of the primer pair for amplification of the *msrA* (A). Primer sequences are also shown (B). *hypA*: hydrogenase expression/formation protein HypA gene; *msrA*: methionine sulphoxide reductase A gene; Cla_0785: hypothetical protein gene.

Nucleotide and deduced amino acid sequences were compared to each other and with accessible sequence data using CLUSTAL W software (1.7 program),²⁴ which was incorporated in the DDBJ.

Southern blot hybridisation analysis

Southern blot hybridisation analysis for the *msrA* gene(s) was carried out using a digoxigenin (DIG)-labelled *msrA* structural gene fragment (approximately 960 bp) prepared from the UPTC CF89-12 strain, as a probe with *Hha* I (TaKaRa Bio, Shiga, Japan) digested whole genomic DNA, according to the procedure described by Sambrook and Russell.²⁵ The fragment probe was amplified using a degenerate primer pair (F3/R2-*msrA*) designed *in silico* (Fig. 1). Random primer extension²⁵ was performed in order to prepare the fragment probe using the Random Primer DNA labelling kit (Ver. 2, TaKaRa Bio Inc.).

Total cellular RNA extraction and purification and RT-PCR

Total cellular RNA was extracted and purified from UPTC NCTC12893 and UPTC CF89-12 cells using the RNeasy Protect Bacteria reagent and RNeasy Mini Kit (Qiagen). The study employed the primer pair F3/R2-*msrA* (Fig. 1) for RT-PCR amplification of the *msrA* gene transcripts segment, and was carried out according to the procedure described previously.²⁶

Synthesis of UPTC MsrA peptide

First, the UPTC MsrA peptide (Cys+GYSGGKPNPSYES, amino acid residues positions 32–44) was synthesised based on the deduced amino acid sequence of the MsrA following nucleotide and amino acid sequence alignment analyses with UPTC NCTC12893 and UPTC CF89-12 MsrAs. This antigenic region was determined using the Lasergene (ver. 9.1, DNASTAR, USA). The N-terminal cysteine was added to facilitate coupling to KLH.

Construction of the expression vector-PCR product ligation complex

Genomic DNA from the UPTC CF89-12 strain was extracted and the *msrA* gene was amplified using the primer pair (f-Cla *msrA*_KpnI, 5'-TTTGGGTACCAATGACAAATAAAGAAATCATTTTAGGTG-3' and r-Cla *msrA*_NotI, 5'-AAAA GCGGCCGCTAGAAATGATTTGCTAGTTTTCTAGC-3'). *Kpn* I and *Not* I restriction sites were added to this primer pair, respectively. After purification using a QIAquick PCR purification kit (Qiagen), the PCR product and expression vector pETIA (BioDynamics Laboratory, Tokyo, Japan) were digested using *Kpn* I and *Not* I. The PCR product was ligated into the pETIA using DNA Ligation Kit Ver. 2.1 (TaKaRa Bio) and transformed into chemically competent *Escherichia coli* JM109, as described by Hanahan.²⁷

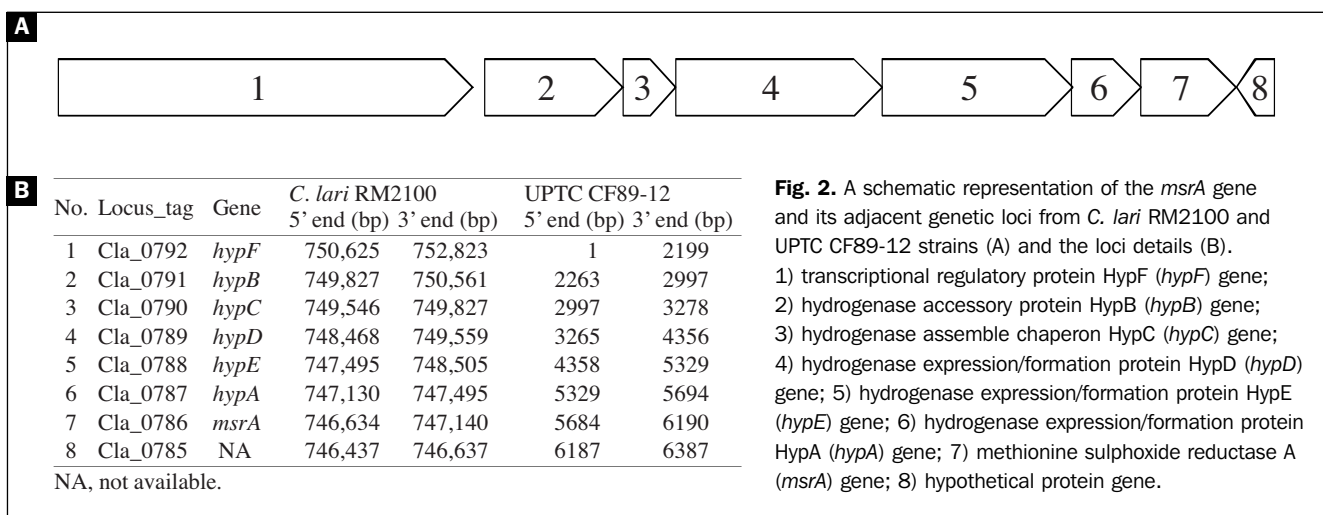


Fig. 2. A schematic representation of the *msrA* gene and its adjacent genetic loci from *C. lari* RM2100 and UPTC CF89-12 strains (A) and the loci details (B). 1) transcriptional regulatory protein HypF (*hypF*) gene; 2) hydrogenase accessory protein HypB (*hypB*) gene; 3) hydrogenase assemble chaperon HypC (*hypC*) gene; 4) hydrogenase expression/formation protein HypD (*hypD*) gene; 5) hydrogenase expression/formation protein HypE (*hypE*) gene; 6) hydrogenase expression/formation protein HypA (*hypA*) gene; 7) methionine sulphoxide reductase A (*msrA*) gene; 8) hypothetical protein gene.

Table 3. Sequence similarities (%) of the nucleotide (upper right) and deduced amino acid (lower left) of the full-length *msrA* structural gene from the 17 *C. lari* isolates and other reference thermophilic campylobacters.

Isolate	1	2	3	4	5	6	7	8	9
1 UPTC NCTC 12893		97.6	98.2	97.6	97.2	97.2	92.0	92.0	92.0
2 UPTC NCTC 12894	94.0		97.8	100	97.4	97.4	92.8	92.8	92.8
3 UPTC NCTC 12895	94.6	94.6		97.8	97.2	97.2	93.2	93.2	93.2
4 UPTC NCTC 12896	94.0	100	94.6		97.4	97.4	92.8	92.8	92.8
5 UPTC CF89-12	94.6	96.4	95.8	96.4		100	92.4	92.4	92.8
6 UPTC CF89-14	94.6	96.4	95.8	96.4	100		92.4	92.4	92.8
7 UPTC A1	91.5	93.3	94.5	93.3	93.9	93.9		100	98.0
8 UPTC A2	91.5	93.3	94.5	93.3	93.9	93.9	100		98.0
9 UPTC A3	93.3	95.1	96.3	95.1	95.7	95.7	98.2	98.2	
10 UPTC 89049	93.3	95.1	96.3	95.1	95.7	95.7	98.2	98.2	100
11 UPTC 92251	91.5	92.7	93.9	92.7	93.3	93.3	98.8	98.8	97.0
12 UPTC 99	95.9	95.8	97.6	95.8	97.0	97.0	93.9	93.9	95.7
13 UN <i>C. lari</i> JCM2530 ¹	95.2	96.4	95.8	96.4	97.6	97.6	93.3	93.3	95.1
14 UN <i>C. lari</i> 176	95.2	96.4	95.8	96.4	97.6	97.6	93.3	93.3	95.1
15 UN <i>C. lari</i> 300	94.6	95.8	96.4	95.8	97.0	97.0	93.3	93.3	95.1
16 UN <i>C. lari</i> 84C-1	95.2	96.4	95.8	96.4	97.6	97.6	93.3	93.3	95.1
17 <i>C. lari</i> RM2100	93.5	94.6	95.2	94.6	95.8	95.8	92.1	92.1	93.9
18 <i>C. jejuni</i> RM1221	62.8	62.8	63.4	62.8	62.2	62.2	64.8	65.4	65.4
19 <i>C. jejuni</i> NCTC11168	62.2	62.2	62.7	62.2	61.6	61.6	64.8	64.8	64.8
20 <i>C. coli</i> RM222816	65.2	62.7	63.4	62.3	63.4	63.4	63.4	63.4	63.4

Production of polyclonal antisera to the *MsrA* synthetic peptide and Western blot analysis

Two rabbits were immunised with 3 mg synthetic peptide that had been emulsified in complete Freund adjuvant. Booster injections of the same dose in saline were given at eight weeks. Serum was obtained before the first injection and at two-week intervals thereafter. Western blot analysis was carried out using the rabbit polyclonal anti-UPTC *MsrA* synthetic peptide antibodies and His-Tag (27E8) mouse

monoclonal antibody (Cell Signaling Technology, Japan), as described previously.²⁸

Results and discussion

Sequence analyses of the *msrA* genes and their adjacent genetic loci

In relation to the *msrA* gene and its adjacent genetic loci



Fig. 3. Nucleotide sequence alignment analyses of non-coding promoter (A) and terminator (B) structure regions for the putative *msrA* gene and the other genes identified in *C. lari* RM2100 and UPTC CF89-12 strains. Dots indicate identical bases; changes are so indicated, and the numbers at the left and right refer to base pairs of the nucleotide positions of the two strains.

10	11	12	13	14	15	16	17	18	19	20
92.4	93.0	98.5	95.8	95.8	96.2	95.8	95.6	71.1	71.3	70.5
93.2	93.4	98.4	96.2	96.2	96.6	96.2	96.0	71.1	70.9	69.7
93.6	93.9	99.0	96.1	96.1	96.8	96.1	96.3	71.6	70.9	70.1
93.2	93.4	98.4	96.2	96.2	96.6	96.2	96.0	71.1	70.9	69.7
92.8	93.0	97.6	96.8	96.8	97.2	96.8	96.6	71.1	70.9	70.1
92.8	93.0	97.6	96.8	96.8	97.2	96.8	96.6	71.1	70.9	70.1
98.0	97.8	92.8	92.0	92.0	92.4	92.0	91.8	71.5	71.5	70.6
98.0	97.8	92.8	92.0	92.0	92.4	92.0	91.8	71.5	71.5	70.6
99.6	97.4	92.8	92.4	92.4	92.8	92.4	92.2	71.8	71.6	69.6
	97.8	93.2	92.8	92.8	93.2	92.8	92.6	71.6	71.4	69.3
97.0		93.4	92.6	92.6	93.0	92.6	92.4	71.7	71.3	70.2
95.7	93.3		96.2	96.2	96.6	96.2	96.0	71.3	71.1	69.9
95.1	92.7	97.0		100	98.8	100	99.0	70.3	70.5	69.7
95.1	92.7	97.0	100		98.8	100	99.0	70.3	70.5	69.7
95.1	92.7	96.4	98.2	98.2		98.8	99.4	70.9	70.7	69.9
95.1	92.7	97.0	100	100	98.2		99.0	70.3	70.5	69.7
93.9	91.5	95.2	98.2	98.2	98.8	98.2		70.7	70.5	69.7
65.4	66.0	62.8	62.2	62.2	63.4	62.2	62.7		97.4	71.8
64.8	65.4	62.2	61.6	61.6	62.7	61.6	62.1	97.6		72.4
63.4	63.4	64.0	64.0	64.0	64.6	64.0	64.0	71.3	71.3	

from *C. lari*, Miller *et al.* identified these (approximately 6300 bp) in the *C. lari* RM2100 strain (NC_012039) by whole genome analysis.²⁹ In the present study, we first showed the schematic representation of the *msrA* structural gene (507 bp) and its adjacent genetic loci from the *C. lari* RM2100 strain (Fig. 2). In addition, during the process of genome

sequence analysis of the Japanese UPTC CF89-12 strain, we identified the sequence (approximately 6300 bp) of the *msrA* structural gene (507 bp) and its adjacent genetic loci, and the resultant schematic representation of those identified in the UPTC CF89-12 strain is also illustrated (Fig. 2), showing that *C. lari* RM2100 and UPTC CF89-12 strains contained the

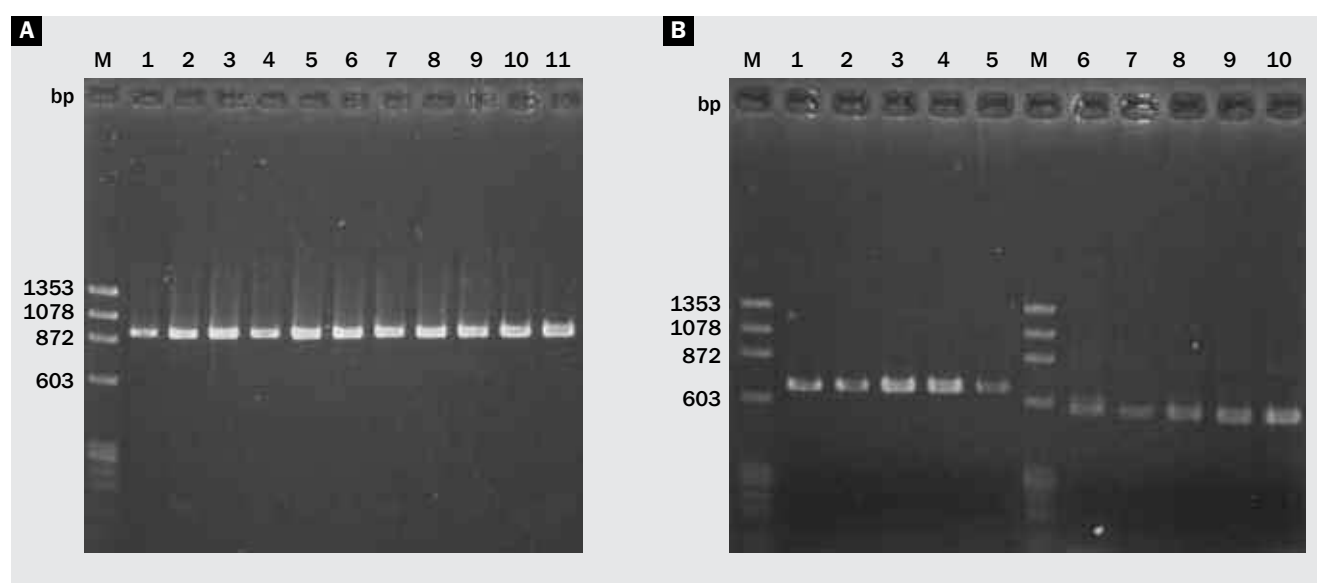


Fig. 4. Electrophoresis profiles of PCR products amplified with 11 *C. lari* isolates using a primer pair of F1-/R1-*msrA* (Lanes 1–11) (A) and with five *C. lari* isolates using primer pairs F1-/R2-*msrA* (Lanes 1–5) and F3-/R5-*msrA* (Lanes 6–10) (B). Lane M : ϕ X174 *Hae* III digest (New England BioLabs). A) Lane 1: UPTC NCTC12893; Lane 2 : NCTC12894; Lane 3: NCTC12895; Lane 4 : NCTC12896; Lane 5 UPTC CF89-12; Lane 6: UPTC CF89-14; Lane 7: UPTC 99; Lane 8: UN *C. lari* JCM2530^T; Lane 9: UN *C. lari* 176; Lane 10: UN *C. lari* 300; Lane 11: UN *C. lari* 84C-1. B) Lanes 1 and 6: UPTC A1; Lanes 2 and 7: UPTC A2; Lanes 3 and 8: UPTC A3; Lanes 4 and 9: UPTC 89049; Lanes 5 and 10: UPTC 92251.

UPTC NCTC12893	1	MTNKEIILGAGCFWCTQAVFDEIKGVVYTEVGVSGGKPNPSYESVINGDGNIEVAKIIYDEKQISLEKILEIFLKMHDPTSLDKQGADKG	90
UPTC NCTC12894	1N.....E.	90
UPTC NCTC12895	1E.	90
UPTC NCTC12896	1N.....E.	90
UPTC CF89-12	1M.....E.	90
UPTC CF89-14	1M.....E.	90
UPTC A1	1V.....E.	90
UPTC A2	1V.....E.	90
UPTC A3	1E.	90
UPTC 89049	1E.	90
UPTC 92251	1V.....V.E.	90
UPTC 99	1E.	90
UN <i>C. lari</i> JCM2530 ^F	1E.....E.	90
UN <i>C. lari</i> 176	1E.....E.	90
UN <i>C. lari</i> 300	1E.....E.	90
UN <i>C. lari</i> 84C-1	1E.....E.	90
<i>C. lari</i> RM2100	1K.....E.....E.	90
UPTC NCTC12893	91	IQYRSVIFYQANEELKTIISDFLQKVQKYYAKAIVTQVLKLEKFYRAEDYHQYFKNNPNQAYCRFIIAPKLEKLANHSNFSF	172
UPTC NCTC12894	91	M.....I.....K.N.....S.T...V.T.....	168
UPTC NCTC12895	91	M.....C.T.....I.....A.....K.N.....S.....V.....	168
UPTC NCTC12896	91	M.....I.....K.N.....S.T...V.T.....	168
UPTC CF89-12	91	V.....T.....I.....K.....S.....V.T.....F----	168
UPTC CF89-14	91	V.....T.....I.....K.....S.....V.T.....F----	168
UPTC A1	91	T.....T.....I.N...A...T.D.....K.N.....S.....V.....VNC-----	166
UPTC A2	91	T.....T.....I.N...A...T.D.....K.N.....S.....V.....VNC-----	166
UPTC A3	91	T.....T.....I.N...A...T.D.....K.N.....S.....V.....VNC-----	166
UPTC 89049	91	T.....T.....I.N...A...T.D.....K.N.....S.....V.....VNC-----	166
UPTC 92251	91	T.....T.....I.N...A...T.D.....K.N.....S.....V.....VNC-----	166
UPTC 99	91	M.....T.....I.....K.....S.....	172
UN <i>C. lari</i> JCM2530 ^F	91	M.....T.....I.....K.....S.....V.T.....	168
UN <i>C. lari</i> 176	91	M.....T.....I.....K.....S.....V.T.....	168
UN <i>C. lari</i> 300	91	M.....T.D...I.....K.....S.....V.T...V.....	168
UN <i>C. lari</i> 84C-1	91	M.....T.....I.....K.....S.....V.T.....	168
<i>C. lari</i> RM2100	91	M.....T.D...I.....K.....S.....V.T...V.....	168

Fig. 5. Deduced amino acid sequence alignment analyses of the possible *msrA* ORFs from 16 *C. lari* isolates ($n=12$ for UPTC; $n=4$ for UN *C. lari*). Dots indicate identical residues; changes are indicated and dashes are deletions; amino acids are designated by the single-letter code. Numbers at the left and right refer to the base pairs of the deduced amino acid sequences, respectively.

msrA structural gene and several adjacent genetic loci in a similar fashion.

Regarding the *msrA* genes and their adjacent genetic loci within these two *C. lari* strains, both appear to be composed of a cluster of the *msrA* gene (Fig. 2, No 7) and the other five genes (Fig. 2, Nos. 2–6). Putative promoter (–35 and –10-like regions) and terminator structures are shown in Figure 3. Those in the UPTC CF89-12 strain are summarised in Table 2. Regarding the transcriptional terminator structure, a putative intrinsic ρ -independent transcriptional terminator structure candidate, which contains a G+C-rich region near the base of the stem and a single-stranded run of T residues, are seen downstream of the stop codon for the *msrA* genes within *C. lari* RM2100 and UPTC CF89-12 strains (Fig. 3B). Thus, the *msrA* gene and its adjacent genetic loci may be very similar within the genomes of *C. lari* organisms, including UPTC. Probable ribosome binding sites (RBS, Shine-Dalgarno sequences)³⁰ that are complementary to highly conserved sequence of CCUCCU close to the 3'-end of 16S ribosomal RNA were identified as an ATGAGG sequence for the *msrA* within the UN *C. lari* RM2100 and UPTC CF89-12 genomic DNA (Fig. 3).

However, the *msrA* genes and their adjacent genetic loci within these two *C. lari* isolates were very different from those of the other thermophilic *Campylobacter* species. An example of *C. jejuni* NCTC11168 was shown to be very different from those of *C. lari*, namely 5'-*aspS* (aspartyl-tRNA synthetase), *adk* (adenylate kinase), *ppa* (inorganic pyrophosphatase), *msrA* and *cj0636* (NOL1/NOP2/sun family protein) -3' within the genomic DNA of the strain (NC_002163).

PCR amplification of the *msrA* gene and its adjacent genetic loci

The PCR primer pair F1/R4-*msrA* (Fig. 1), which ought to generate a product of approximately 900 bp of the *msrA* gene and its adjacent genetic loci in length was found to amplify a product of the similar size with the 11 *C. lari* isolates (Fig. 4A). The PCR primer pairs F1/R2-*msrA* and F3/R4-*msrA*, which ought to generate products of approximately 656 bp and 593 bp, were also found to amplify products of a similar size with the five *C. lari* isolates (Fig. 4B). In the present study, the purified PCR products were subjected to cycle sequencing and then their nucleotide and deduced amino acid sequences were determined.

Nucleotide and deduced amino acid sequences of the *msrA* gene and its adjacent genetic loci

The nucleotide sequences of the amplicons and the deduced amino acid sequences of the possible open reading frame (ORF) of the *msrA* gene from the 16 *C. lari* isolates ($n=12$ UPTC, $n=4$ UN *C. lari*) are accessible with the accession numbers shown in Table 1. The nucleotide sequences of the possible ORF were found to be 498–516 bp, equivalent to the nucleotide positions (np) 5684–6187 bp for UPTC CF89-12 (AB702689), for the *msrA* gene from the 16 *C. lari* isolates and *C. lari* RM2100 strain (data not shown).

Deduced amino acid sequence alignment analysis was then carried out to elucidate differences in MsrA protein among the 17 *C. lari* isolates. As shown in Figure 5, less than 30 amino acid positions were identified to differ from each other among the 17 possible MsrA ORFs. In addition, these

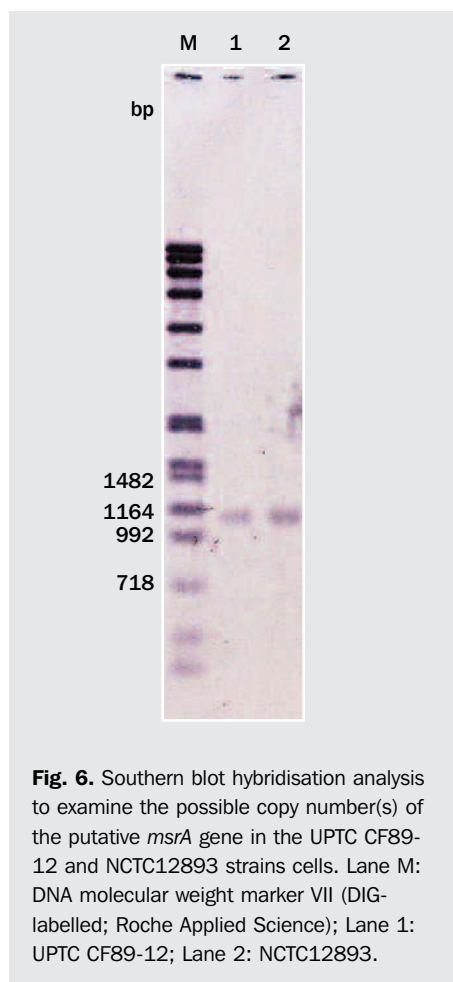


Fig. 6. Southern blot hybridisation analysis to examine the possible copy number(s) of the putative *msrA* gene in the UPTC CF89-12 and NCTC12893 strains cells. Lane M: DNA molecular weight marker VII (DIG-labelled; Roche Applied Science); Lane 1: UPTC CF89-12; Lane 2: NCTC12893.

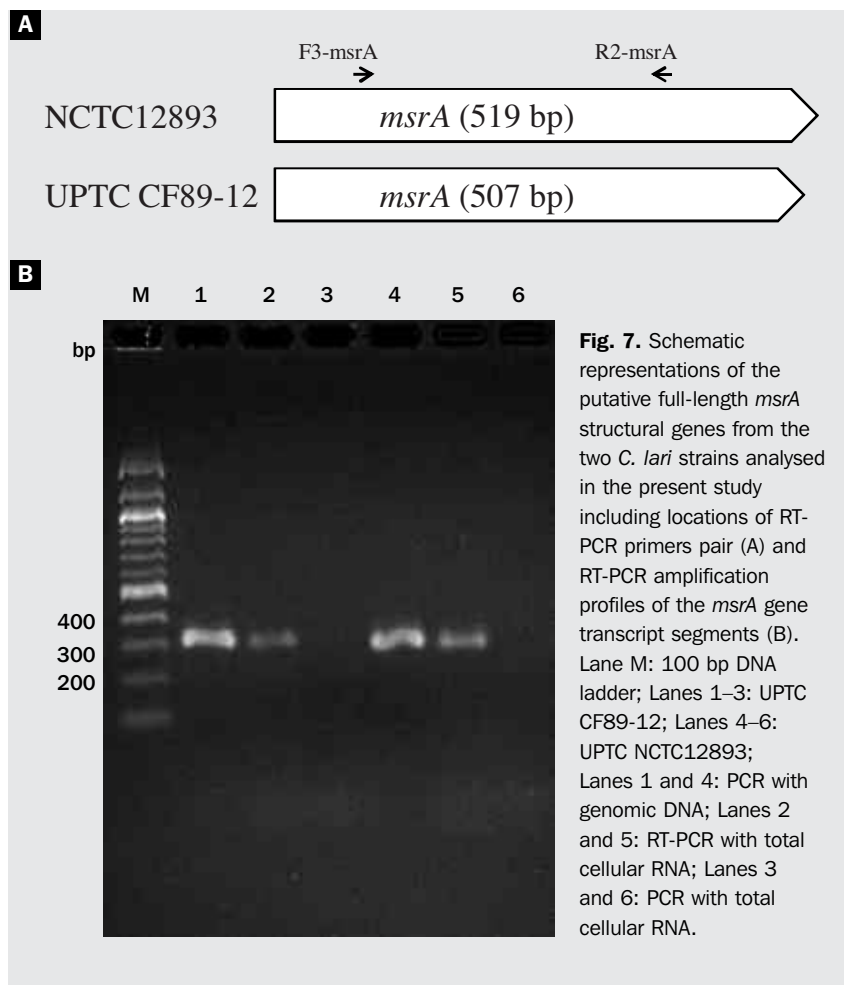


Fig. 7. Schematic representations of the putative full-length *msrA* structural genes from the two *C. lari* strains analysed in the present study including locations of RT-PCR primers pair (A) and RT-PCR amplification profiles of the *msrA* gene transcript segments (B). Lane M: 100 bp DNA ladder; Lanes 1–3: UPTC CF89-12; Lanes 4–6: UPTC NCTC12893; Lanes 1 and 4: PCR with genomic DNA; Lanes 2 and 5: RT-PCR with total cellular RNA; Lanes 3 and 6: PCR with total cellular RNA.

were predicted to encode peptides with the calculated molecular weights (CMWs) of approximately 19.0–19.8 kDa. Thus, regarding the *msrA* genes from the *C. lari* isolates examined, similarly complete ORF and RBS structures were shown to exist.

Nucleotide and deduced amino acids sequences similarities

Nucleotide sequences of the full-length *msrA* genes from the 17 *C. lari* isolates including *C. lari* RM2100 showed 91.8–100% sequence similarities to each other and 69.3–71.8% similarities to those of two *C. jejuni* and one *C. coli* strains, as shown in Table 3. In addition, the possible ORFs of the *msrA* gene from the 17 *C. lari* isolates showed 91.5–100% amino acid sequence similarities to each other, and 61.6–66.0% similarities to those of the three strains, as shown in Table 3. These indicated that the nucleotide and deduced amino acid sequence similarities of the *msrA* structural genes and their possible ORFs were very close among the *C. lari* species isolates including UPTC taxon.

Southern blot hybridisation analysis

An attempt was made to examine the possible copy number(s) of the putative *msrA* gene in UPTC CF89-12 and NCTC12893 using Southern blot hybridisation analysis. As shown in Figure 6, *Hha* I-digested whole genomic DNA prepared from the UPTC CF89-12 and NCTC12893 strains each gave one positive hybridisation signal, suggesting that

the strain may carry one putative *msrA* gene or homologue within their genomic DNA.

RT-PCR analysis of the *msrA* gene transcripts

RT-PCR results using the primer pair of F3/R2-*msrA* clearly indicated the *msrA* gene could be expressed at the transcriptional level in the UPTC CF89-12 and NCTC12893 cells (Fig. 7), and thus the *msrA* gene is apparently transcribed in UPTC CF89-12 and NCTC12893.

Western blot analysis of cell extracts

When Western blot analysis was carried out to clarify whether or not MsrA could be identified in the cell extracts from the *C. lari* isolates, it was shown that anti-UPTC MsrA synthetic peptide antibodies identified an immunoreactively positive signal in both the UPTC CF89-12 and NCTC12893 strains examined (Fig. 8).

In conclusion, this is the first demonstration of the molecular cloning and characterisation of the *msrA* gene locus in *C. lari* organisms, using several degenerate PCR primer pairs designed *in silico*, and also *msrA* sequence gene expression at the transcriptional and translational levels. □

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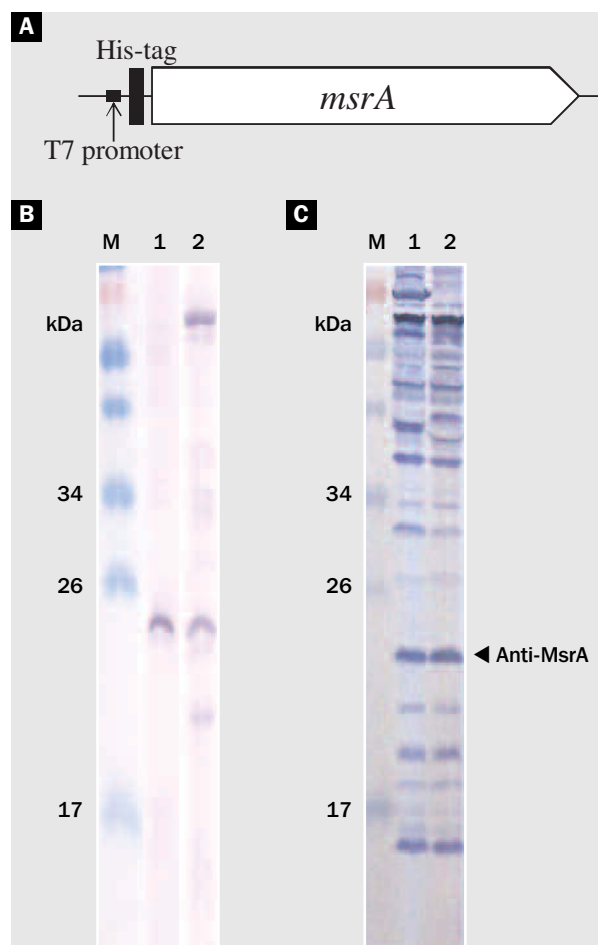


Fig. 8. Western blot analysis of the cell extracts from UPTC isolates using the rabbit polyclonal anti-UPTC MsrA synthetic peptide antibodies (C). A schematic representation of the expression vector is shown (A) and the expression vector pETIA-MsrA ligation complex was detected using the rabbit polyclonal anti-UPTC MsrA synthetic peptide antibodies (Lane 1) and His-Tag (27EA) Mouse mAb (Lane 2) (B). B,C) Lane M: Page Ruler Prestained Protein Ladder (Fermentas Life Sciences, Tokyo, Japan). C) Lane 1: UPTC CF89-12; Lane 2: UPTC NCTC12893.

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