First isolation and molecular characterisation of a cryptic plasmid from urease-negative *Campylobacter lari*

H. BATORI, T. SEKIZUKA, O. MURAYAMA, J. E. MOORE*, B. C. MILLAR* and M. MATSUDA

*Laboratory of Molecular Biology, School of Environmental Health Sciences, Azabu University, Fuchinobe 1-17-71, Sagamihara 229-8501, Japan; and *Department of Bacteriology, Northern Ireland Public Health Laboratory, Belfast City Hospital, Belfast BT9 7AD, N Ireland, UK*

Campylobacter lari is a relatively new thermophilic Campylobacter species.1,2 Five years after the first identification of C. lari, urease-positive thermophilic campylobacters (UPTC) were isolated from the natural environment in England in 1985.3 The characterisation of UPTC as a variant or a biovar of C. lari has been described.4,5 Thus, two representative taxa, namely urease-negative (UN) thermophilic C. lari and UPTC, occur within the C. lari species.

So far, the detection, isolation and characterisation of plasmids of *C. jejuni* and *C. coli*, which are the two most common pathogenic thermophilic campylobacters, have frequently been found to be the causative agents of infection in humans and animals.[6–13]

However, no other descriptions of the plasmid analysis of *C. lari* appear to have been reported, other than this group's previous publication of plasmid profiles of UPTC isolates in Europe and Asia (Japan).[14] Therefore, the aim of the present study is to identify the presence of cryptic plasmids in isolates of UN *C. lari* and subsequently clone, sequence and analyse such plasmids.

The UN *C. lari* 300 isolate analysed in the present study was obtained from a seagull in the USA,[15] and cells were cultured as described previously.[14] Plasmid DNA was isolated by two methods, described by Birnboim and Doly[16] and Kado and Liu.[17] Isolated plasmid (designated pCL300) DNA was subjected to 0.7% (w/v) agarose gel electrophoresis in 0.5 x TBE (0.09 mol/L Tris, 0.09 mol/L borate, 2 mmol/L EDTA). Purified pCL 300 DNA from the UN *C. lari* 300 isolate was then digested with the restriction enzyme, *Hind* III (Toyobo, Osaka, Japan), following screening experiments with other restriction enzymes (data not shown).

The resultant restricted fragments after alkaline phosphatase treatment were then ligated into the *Hin*d III-digested pUC19 vector. The ligated recombinant DNA was transformed into competent *Escherichia coli* DH5α cells, using the procedure described by Sambrook *et al*.[18] The transformants were selected on LB agar containing ampicilin (50 μg/mL), X-gal (40 μg/mL) and IPTG (0.1 mmol/L). White colonies were subcultured on LB-ampicilin agar. The resultant recombinant plasmids containing the desired fragments were obtained.

Initially, five positive pUC19 plasmid clones containing *Hin*d III-digested pCL300 fragments were obtained, and those pUC19 plasmid DNAs were extracted by an alkaline sodium dodecyl sulphate (SDS) purification procedure. The recombinant plasmid DNAs were sequenced using a Texas Red-labelled primer pair. Sequencing of the cloned pCL300 DNA was performed in an Hitachi DNA autosequencer (SQ5500EL, Hitachi, Tokyo, Japan). Sequence analysis was performed using GENETYX-MAC (version 9) computer software (GENETYX, Tokyo, Japan).

The nucleotide sequence data of the plasmid pCL300 DNA determined in the present study are accessible in the DDBJ/EMBL/GenBank (AB211496). A nucleotide sequence of approximately 3.6 kbp of pCL300 from the UN *C. lari* isolate studied was compared to accessible sequence data of other thermophilic *Campylobacter* plasmids, using CLUSTAL W software (1.7 program),[19] which is incorporated in DDBJ. A phylogenetic tree was constructed by an unweighted pair group method using arithmetic means (UPGMA) available on the GENETYX-MAC program.

Initially, the study screened for the presence of plasmid DNA in a total of 29 UN *C. lari* isolates from the natural environment, including wild birds, food and humans, in Canada, Northern Ireland, Japan and the USA (Table 1). Screening results indicated the presence of plasmid DNA in only one (UN *C. lari* 300) of the 29 isolates, at a frequency of approximately 3.4%. This value is characteristically very low compared with the frequencies of approximately 33% for *C. jejuni* and *C. coli*[7] and 30% for UPTC.[14]

Based on the analysis of the nucleotide sequence determined in the present study, the plasmid DNA from UN *C. lari* 300 is a circular and double-stranded molecule of 3634 bp with a G+C content of 28.7%, which is lower than the overall G+C content (30–34%) of the thermophilic *Campylobacter* genome DNA.[2]

In the 3634 bp of pCL300 DNA, a total of 10 possible open reading frames (ORFs) (nucleotide position [np] 3511 [or 129] -1082, np 1576–1629, np 1708–2739, np 2764–3136, np 2895–3146 and np 3311–3524 on a strand, and np 141–260, np 431–508, np 1618–1731 and np 2765–3103 on another complementary strand, which contained both a start and a termination codon, respectively), occurred. However, only four possible ORFs were found to give a sequence similarity with any known genes (proteins), and to carry any promoter-like sequences and ribosome binding (RB) sites upstream, as well as transcriptional terminator-like sequences, based on the sequence alignment and analysis.

A schematic representation of the four possible ORF maps of *C. lari* pCL300 and *Hin*dIII and some other restriction sites are shown in Figure 1. In the present study, the first A in the initial *Hin*dIII recognition sequence (AAGCTT; the numbers are shown in parentheses) is designated np 1.

As with the other plasmid DNAs from Gram-negative bacteria reported, the present pCL300 DNA also contained an A+T-rich region (np 1200–1321; A+T content of 87.7%) followed by multiple direct tandem repeat units of 22 bp (5′-TATTAAGGGGGCAAATCTAAAC-3′; np 1361–1485), characteristic of a replication origin (iteron sequence).[12]

In pCL300, the iteron contains four complete repeat units of 22 bp and one partial unit of 15 bp (5′-TATTAAGGGGGCAAA-3′). The present iteron sequence of pCL300 showed significant sequence similarity to other iteron sequences reported previously for thermophilic *Campylobacter* pCJ419 (AY256846), pCJ01 (AF301164), pCCT1 (X82079) and pCCT2 (X82080) (Fig. 2).

Correspondence to: Dr. Motoo Matsuda
Email: matsuda@azabu-u.ac.jp

Table 1. Isolates of UN *C. lari* analysed in this study (NA: not available; ND: not detected).

Isolate no.	Campylobacter	Source	Country	Plasmid
28	UN *C. lari*	Mussel	N. Ireland	ND
34	UN *C. lari*	Food	N. Ireland	ND
48	UN *C. lari*	Mussel	N. Ireland	ND
82 C-1	UN *C. lari*	Human	N. Ireland	ND
82 C-2	UN *C. lari*	Human	N. Ireland	ND
99	UN *C. lari*	Sea water	N. Ireland	ND
155	UN *C. lari*	Food	N. Ireland	ND
170	UN *C. lari*	Seagull	Japan	ND
175	UN *C. lari*	Black-tailed gull	Japan	ND
176	UN *C. lari*	Black-tailed gull	Japan	ND
177	UN *C. lari*	NA	NA	ND
254	UN *C. lari*	Food	N. Ireland	ND
264	UN *C. lari*	Mussel	N. Ireland	ND
274	UN *C. lari*	Mussel	N. Ireland	ND
277	UN *C. lari*	Seagull	Japan	ND
288	UN *C. lari*	Black-tailed gull	Japan	ND
293	UN *C. lari*	Seagull	Japan	ND
296	UN *C. lari*	Human	Canada	ND
298	UN *C. lari*	Human	Canada	ND
299	UN *C. lari*	Human	U.S.A	ND
300	UN *C. lari*	Seagull	U.S.A	detected
302	UN *C. lari*	NA	NA	ND
381	UN *C. lari*	Mussel	N. Ireland	ND
382	UN *C. lari*	Mussel	N. Ireland	ND
448	UN *C. lari*	Mussel	N. Ireland	ND
2316A3	UN *C. lari*	NA	NA	ND
JCM2530T	UN *C. lari*	NA	Japan	ND
TCM2536	UN *C. lari*	NA	NA	ND
35221	UN *C. lari*	NA	NA	ND

A possible ORF-1 (np 3511–1082) in pCL300 DNA was located upstream of the A+T-rich region and the iteron seqeuence (Fig. 1), and encodes a 318 amino acid protein that gave a 70% nucleotide sequence similarity to a part of the mobilisation (Mob) protein of the plasmid pCJ419 from *C. jejuni* described previously (AY256846).[12] Alternatively, another possible ORF-1, encoding a 407 amino acid protein that gave 46% amino acid sequence similarity to part of the protein described above, may occur between np 129 and 1082 in the plasmid. In relation to the promoter structure for the two possible hypothetical ORF-1s, two types of putative promoter-like structure (np 3471–3510 and np 89–128) were demonstrated, based on the nucleotide sequence alignment analysis shown in Figure 3. Probable RB sites complementary to a highly conserved sequence of CCUCCU close to the 3′ end of 16S ribosomal RNA were not detected for the ORF-1s.

Furthermore, there is a hypothetical transcriptional terminator-like structure (np 1116–1138) that can form a hairpin loop structure containing a G+C-rich region near the base of the stem and a single stranded run of U residues downstream of the ORF-1. However, whether or not these ORF-1s express the Mob protein remains unclear.

Two possible ORFs (ORF-2 and ORF-3) that encode putative replication initiation proteins were located immediately downstream of the iteron sequences (Fig. 1). A possible ORF-2 (np 1576–1629) is a short ORF encoding a polypeptide of 18 amino acids that gave 61% amino acid sequence similarity to a putative RepA protein of the plasmid pCJ419 of *C. jejuni* (AY 256846).[12] A possible ORF-3 (np 1708–2739) encodes a protein of 344 amino acids that gave 72% amino acid sequence similarity to a putative RepB protein of the plasmid pCJ419.[12] In addition, another possible ORF-4 that encodes a protein of 84 amino acids and gave 64% amino acid sequence similarity to part of a hypothetical protein of unknown function, Cjp32[12] occurred between np 2895 and 3146 (Fig. 1). No promoter-like structures were demonstrated for ORF-3 and ORF-4.

Two putative promoter structures, consisting of consensus sequences at -35 region (TTGAGT; np 1532–1537) and -10 region (CATAAC, Pribnow box; np 1558–1563), as well as the start codon (np 1576–1579) for a possible ORF-2, were demonstrated for a typical transcriptional promoter, immediately upstream of the possible ORF-2 (a putative RepA). Three putative RB sites of AGGA (np 1566–1569) for the possible ORF-2 (RepA), AGGA (np 1698–1701) for the

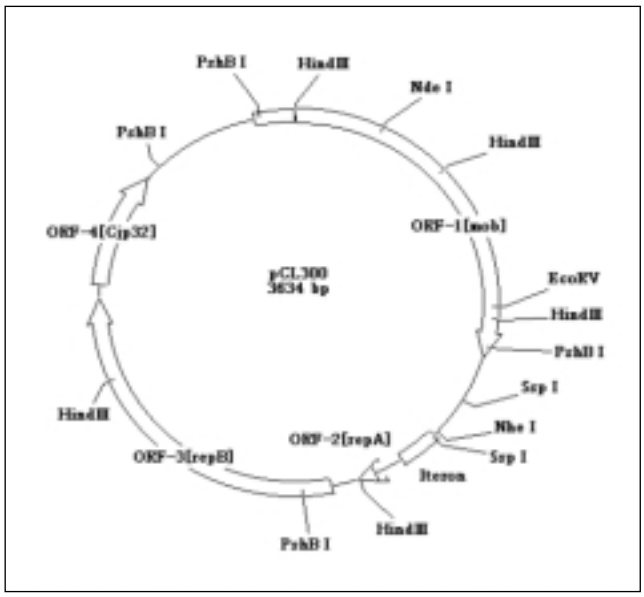

Fig. 1. A schematic representation of the four possible ORF maps of *C. lari* pCL300 and *Hin*dIII and some other restriction sites.

Fig. 2. The iteron sequence of pCL300 showed significant sequence similarity to other iteron sequences reported previously for thermophilic campylobacters.

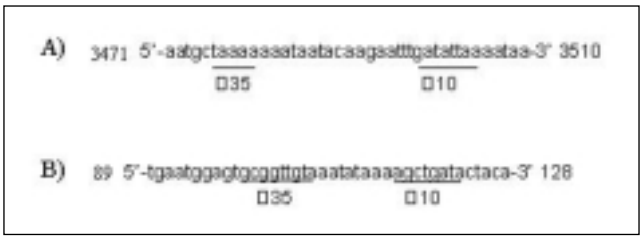

Fig. 3. Two types of the putative promoter-like structures for the two hypothetical ORF-1s. Numbers at each end refer to base pairs of the nucleotide sequences of the pCL300.

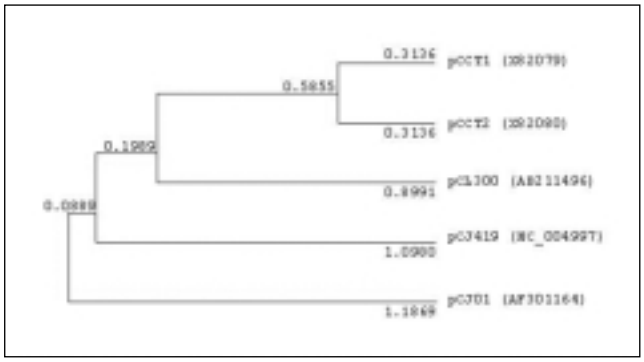

Fig. 4. Major clusters are apparent in a phylogenetic tree constructed using the UPGMA method.

possible ORF-3 (RepB) encoding 344 amino acids, and ATGGGG (np 2878–2883) for the possible ORF-4 were also demonstrated. A hypothetical intrinsic transcription terminator structure that contains a G+C-rich region, near the base of the stem and a single-stranded run of U residues, was demonstrated downstream of ORF-4 (np 3208–3236). Thus, ORF-2, -3, and -4 may constitute a polycistronic operon and, therefore, generate a single messenger RNA (mRNA) transcript. However, whether or not this polycistronic operon suggested in the pCL300 in the present study is functional remains unclear.

In relation to the nucleotide sequence similarity analysis, whole pCL300 DNA gave 61.8% sequence similarity to the DNA of pCJ419, 58.1% to pCJ01, 57.8% to pCCT2 and 55.7% to pCCT1. A phylogenetic tree constructed using the UPGMA method is shown in Fig. 4, and some major clusters are apparent. Moreover, pCJ419 was demonstrated to be the nearest neighbour to pCL300, which may suggest shared ancestry.

Shortly after completing the work reported here, Fouts *et al*.[20] described a single plasmid pCL46 isolated from a *C. lari* RM2100 strain, approximately 46 kb in length, which contained 45 ORF numbers. However, to date, no detailed information is available on these ORFs.

Antibiotic resistance is now a major problem with thermophilic campylobacters (*C. jejuni* and *C. coli*), in the treatment of both animals and humans.[21] However, it is interesting to note from the work reported here that the frequency of isolation of plasmids from UN *C. lari* is very low, which perhaps indicates a lack of antibiotic selective pressure exerted on the isolates examined in this study, due to their origins in the natural environment.

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Differentiation of vancomycin-resistant enterococci using enterococcus differential medium

R. E. NICHOLAS, T. G. WINSTANLEY, E. J. RIDGWAY and J. D. PERRY*

Department of Microbiology, Royal Hallamshire Hospital, Sheffield; and
**Department of Microbiology, Freeman Hospital, Newcastle upon Tyne, UK*

The genus *Enterococcus* includes over 20 species.[1] Increasing numbers of enterococci are becoming resistant to glycopeptide antibiotics. *Enterococcus faecium* and, to a lesser extent, *E. faecalis* are of greater epidemiological importance,[2] as they harbour transferable *vanA* and *vanB* genes. Other enterococci such as *E. gallinarum* and *E. casseliflavus / E. flavescens* do not normally cause human disease but commonly demonstrate intrinsic low-level glycopeptide resistance.[3] Furthermore, they are often isolated as a result of active surveillance procedures.

Knowledge of the type of resistance is critical for infection control purposes and, in a non-molecular laboratory, necessitates accurate identification. Accurate identification is also required to determine whether changes in glycopeptide resistance rate reflect the dissemination of resistance determinants among enterococci in general or a rise in the relative importance of *E. faecium*, the strain in which glycopeptide resistance is most common.

The Health Protection Agency (HPA) standard method for identification of enterococci[4] recommends the use of 'a commercial kit'. Along with the API Rapid ID 32 Strep (bioMerieux, Basingstoke, UK), BBL Crystal Gram Positive (Becton Dickinson, Oxford, UK) and BBL Crystal Rapid Gram Positive (Becton Dickinson), the API 20 Strep system (bioMerieux) is often used to identify enterococci to species level. However, Reed *et al*.[5] used API 20 Strep and misidentified nine out of 12 *E. gallinarum* isolates as *E. faecium*. Similarly, Winston *et al*.[6] used this system to identify 46 enterococcal isolates with low-level vancomycin resistance (predominantly *E. gallinarum*) and misidentified 42 as *E. faecium*. The correct identification was obtained only when additional tests were employed.

E. faecium and *E. faecalis* are non-motile, whereas *E. gallinarum* and *E. casseliflavus / E. flavescens* are considered to be motile.[7] Furthermore, most isolates of *E. casseliflavus / E. flavescens* have a distinct yellow pigment. Collins *et al*. [8] used acid production from methyl-α-D-glucopyranoside (MGP) to identify certain streptococci as enterococci. Devriese *et al*.[9] confirmed that the acidification of MGP differentiated *E. casseliflavus* and *E. gallinarum* from *E. faecium*. Turenne *et al*.[7] examined 33 isolates identified as *E. faecium* by conventional methods and showed that, of the 11 that sequenced as *E. casseliflavus* or *E. gallinarum*, all were MGP-positive, while the *E. faecium* were MGP-negative. The MGP test has been evaluated by workers in Brazil,[10] the USA[11] and Canada,[12] with similar results.

Correspondence to: R. E. Nicholas, Department of Microbiology, Royal Hallamshire Hospital, Sheffield, S10 2JF, UK
Email: rachelle.e.nicholas@sth.nhs.uk