Cloning, sequencing and molecular characterisation of a cryptic plasmid from a urease-positive thermophilic *Campylobacter* (UPTC) isolate

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ABSTRACT

Cloning, sequencing and molecular characterisation of a cryptic plasmid, pUPTC237, from a urease-positive thermophilic *Campylobacter* (UPTC) isolate obtained from the natural environment in Northern Ireland is reported in this study. Based on the determined DNA sequence, the pUPTC237 DNA was identified as a circular molecule of 3828 bp with a G+C content of 29.5%. As with other plasmid DNAs from Gram-negative bacteria, pUPTC237 contained an A+T-rich region (A+T content: 95%), followed by multiple direct tandem repeat units of 22 bp, characteristic of a replication origin and iteron sequence. A possible open reading frame (ORF)-1 was located upstream of the A+T-rich region and the iteron sequence that encoded a 460 amino acid protein similar to the mobilisation (mob) protein and two putative promoter structure sequences at the -35 and -10 regions and a possible ribosome binding site occurred upstream of the start codon for the ORF-1. Moreover, three possible ORFs (a short ORF-2 encoding 26 amino acids, similar to repA; an ORF-3 encoding 341 amino acids, similar to repB; and an ORF-4 encoding 96 amino acids with unknown function) were also identified. There are also two putative promoter structures for these three ORFs at the -35 and -10 regions upstream of the possible ORF-2. A possible transcription termination region was identified downstream of ORF-4. Northern blot hybridisation analysis suggested that these four ORFs constitute an operon and generate a messenger RNA (mRNA) transcript.

KEY WORDS: Blotting, Northern.
Campylobacter.
Molecular sequence data.
Plasmids.

Introduction

Campylobacter lari is a thermophilic species demonstrated to be resistant to nalidixic acid, one of the compounds generally used to disciminate this species from the two main thermophilic *Campylobacter* spp., *C. jejuni* and *C. coli*.[1,2] *C. lari* has been isolated mainly from seagulls of the genus *Larus*[1–4] and has been found occasionally as a cause of clinical infection.[5–10]

Five years on from the first identification of *C. lari* by Skirrow and Benjamin in 1980,[1] urease-positive thermophilic *Campylobacter* (UPTC) organisms were isolated from the natural environment in England,[11] and subsequent human isolates of UPTC have been reported in France.[12,13] The characterisation of UPTC as a variant or a biovar of *C. lari* has been described.[12,14] Additional isolates of UPTC have been collected from the natural environment and from fresh faecal specimens of seagulls in Northern Ireland[15–18] and in The Netherlands,[19] and UPTC isolates have also been reported in Japan.[20,21] Thus, two representative taxa, urease-negative (UN) thermophilic *Campylobacter lari* and UPTC,[22] occur within the species of *C. lari*.

To date, the isolation, sequencing and characterisation of plasmids of the most representative thermophilic *C. jejuni* and *C. coli* have been reported.[23–27] In relation to the plasmid analysis of *C. lari*, the authors have reported the first isolation and molecular characterisation of a cryptic plasmid from urease-negative *C. lari*.[28] To the authors' knowledge, however, no other descriptions of plasmid analysis of UPTC, a representative taxon of *C. lari*, have appeared, although they previously described plasmid profiles of UPTC strains isolated in Europe and in Japan.[29]

The aim of the present study, therefore, is to clone, sequence and undertake molecular characterisation of a plasmid from a UPTC isolate.

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Materials and methods

The UPTC237 isolate analysed in the present study was obtained from an oyster collected in Northern Ireland.[18] Cells were cultured as described previously.[29] Plasmid DNA was isolated according to the method described by Birnboim and Doly.[30] Isolated plasmid (designated as pUPTC237) DNA was subjected to agarose (0.7% [w/v]) gel electrophoresis in 0.5 x TBE (0.09 mol/L Tris, 0.09 mol/L borate, 2 mmol/L EDTA [pH 8.3]). Purified pUPTC237 DNA from the UPTC237 isolate

was digested with *Hin*d III (approximately 1500, 1400 and 1100 bp) and *Mbo*l (approximately 2900, 700 and 500 bp) (Toyobo, Osaka, Japan), according to the manufacturer's instructions.

The restricted fragments obtained after alkaline phosphatase treatment were ligated into *Hin*dIII- and *Bam*H1-digested pUC19 vectors, respectively. The ligated recombinant DNA was transformed in competent *Escherichia coli* DH5 α, according to the procedure described by Sambrook *et al*.[31] The transformants were selected on LB agar containing ampicillin (50 μg/mL), X-gal (40 μg/mL) and IPTG (0.1 mmol/L).

White colonies were subcultured on LB-ampicillin agar. The resultant recombinant plasmids containing the desired fragments were obtained. Initially, positive pUC19 plasmid clones were obtained that contained *Hin*d III- and *Mbo*l-digested pUPTC237 fragments. These pUC19 plasmid DNAs were extracted using an alkaline sodium dodecyl sulphate purification procedure.

The recombinant plasmid DNAs were sequenced using a Texas red-labelled primer pair. Sequencing of the cloned pUPTC237 DNA was performed using a Hitachi DNA autosequencer (SQ5500EL, Hitachi Electronics Engineering, Tokyo, Japan). Sequence analysis was performed using the GENETYX-MAC (version 9) computer software (GENETYX, Tokyo, Japan).

Nucleotide sequence data of the plasmid pUPTC237 DNA from the UPTC237 isolate determined in the present study are accessible in the DDBJ/EMBL/GenBank (accession number: AB 256957).

Total RNA was extracted and purified from the UPTC237 isolate cells containing the pUPTC237 plasmid. Total RNA was also extracted and purified from the two UPTC isolates (UPTC2 and UPTC476),[29] which did not contain plasmid DNA. RNA was separated by electrophoresis in agarose (1.0% [w/v]) gel containing 2.2 mol/L formaldehyde with 1 x MOPS (20 mmol/L MOPS [pH 7.0]). Northern blot hybridisation was carried out according to the procedure described by Sambrook *et al*.[31] The present study used the *mob* fragment amplified by a primer pair of *mob*-f (5′-ACCGCCTAGTTATTTGATCGGC-3′) and *mob*-r (5′-CTTTAGAATGCTCGGGTGGTC-3′), and the *repB* fragment amplified by a primer pair of *repB*-f (5′-GGACTTGCTTTGCGGT-3′) and *repB*-r (5′-CGCACCTGATCAAACAAG-3′) as probes, in order to confirm the expression of pUPTC237 DNA operon in the host UPTC237 isolate cells. Random primer extension was performed in order to prepare the non-radioactive digoxigenin-labelled DNA probe.[31]

Results

Based on the nucleotide sequence data determined in the present study, pUPTC237 DNA is a circular molecule of 3828 bp with a G+C content (approximately 29.5%) that is lower than the overall G+C content (30–34%) of the thermophilic *Campylobacter* genome DNA.[2] Of the four possible ORFs in the 3828 bp of pUPTC237 DNA, three were found to give sequence similarities with some known proteins, and one was found not to give any similarity, based on the sequence alignment and analysis data.

A schematic representation of a map of the four possible ORFs of pUPTC237 and *Hin*dIII and *Mbo*l restriction sites is shown in Figure 1. At present, the first A in the No. 1 *Hin*dIII recognition sequence (AAGCTT) is designated as nucleotide position (np) 1, as described by Batori *et al*.[28]

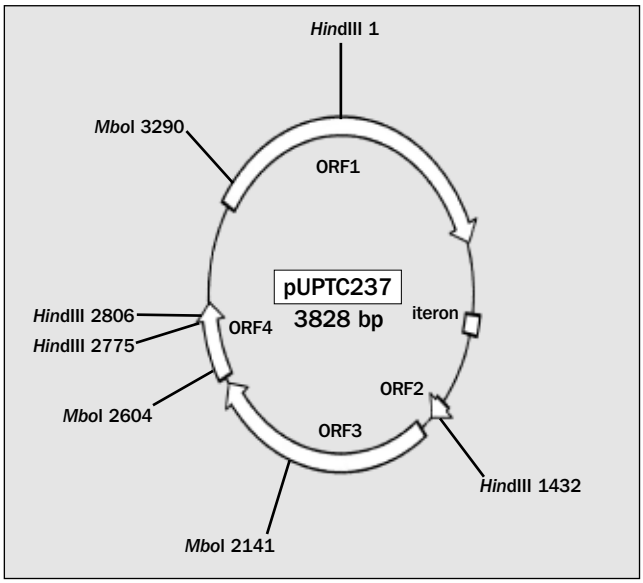

Fig. 1. A schematic representation of a map of the four possible ORFs of pUPTC237 and *Hin*dIII and *Mbo*l restriction sites.

As with other reported plasmid DNAs from Gram-negative bacteria, the present pUPTC237 DNA contained an A+T-rich region (np 937–977; A+T content: 95%) followed by multiple direct tandem repeat units of 22 bp (5′-TATTAAAAGTAGAAATTTAAAC-3′; np 1017–1103), characteristic of a replication origin (iteron sequence).[25] In pUPTC237, the iteron contains three complete repeat units of 22 bp and one partial unit of 21 bp (5′-TATTAAAAGTAGAAATTTAAA-3′). The present iteron sequence of pUPTC237 is similar, but not completely identical, to other plasmid iteron sequences from thermophilic campylobacters reported previously (Fig. 2).

A possible ORF-1 (np 3204–755) in pUPTC237 DNA was located upstream of the A+T-rich region and the iteron sequence, which encoded a 460 amino acid protein that gave a 58.2% nucleotide sequence similarity to a putative mobilisation (mob) protein of the plasmid pCJ419 from *C. jejuni* described previously (GenBank accession number: NC_004997)[25] and 66.9% to pCL300 from *C. lari* (AB211496).[28]

Two possible ORFs, ORF-2 and ORF-3, which encode

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pUPTC237    -T-A--TTAAAA-GTA-GAAATT-TAAAC
pCL300      -.-.--...-.GG...-....-.GGT.GG
pCJ419      -.-.--...-..A...-......GGG.-G
pCJ01       -.-.--...-.GG...-....-.GGT.GG
pCCT1       -.-.--.....C.-.CA....C.--..
pCCT2       -.-.--...-.GG...-C...-.TGT.GG
p3384       -.-.--.....C.-.CA....C.--..
p3386       G.G.TT...T..--.-C-T....-.--.-
              * *  *** *         ***
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Fig. 2. An iteron sequence of pUPTC237 showing similarity to other plasmid iteron sequences from thermophilic campylobacters reported previously.

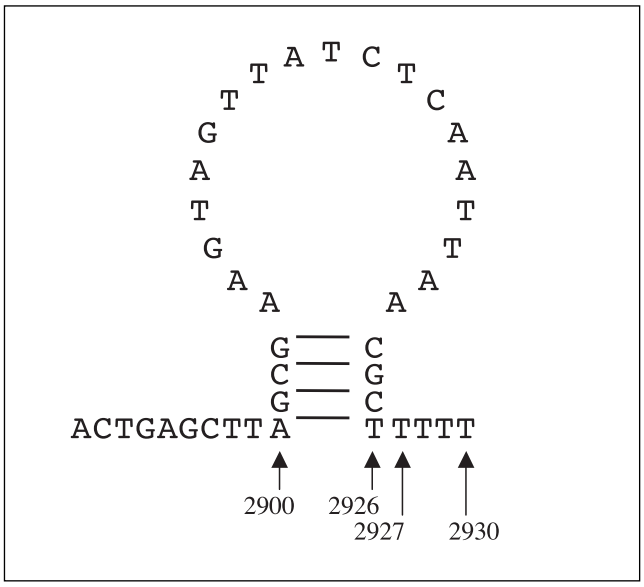

Fig. 3. A hypothetically intrinsic transcription terminator structure, which contains a G+C-rich region near the base of the stem (np 2900–2926) and a single-strand run of U (np 2927–2930) residues, was demonstrated downstream of the ORF-4.

putative replication (rep) proteins, were located immediately downstream of the iteron sequences. A possible ORF-2 (np 1378–1455) is a short possible ORF encoding a protein of 26 amino acids that gave an approximately 90% nucleotide sequence similarity to a putative repA protein of the plasmid pCJ419 (NC_004997)[25] and 74.5% to pCL300 (AB211496). A possible ORF-3 (np 1511–2533) encodes a protein of 341 amino acids that gave about 66.5% nucleotide sequence similarity to a putative repB protein of the plasmid to pCJ419 (NC_004997) and 75.7% to pCL300 (AB211496). Another possible ORF-4 occurred between np 2553 and np 2840 that encodes a protein of 96 amino acids, and this showed no nucleotide sequence similarity with other proteins of unknown function whose sequence data are accessible in the DDBJ/EMBL/GenBank.

In relation to the promoter structure for these four possible ORFs, two putative promoter structures, consisting of consensus sequences at the -35 region (TGCCGA; np 3154–3158) and -10 region (AATAAT, Probnow box; np 3181–3186), as well as the start codon for the possible ORF-1 (np 3204–3206), were identified as typical transcriptional promoters immediately upstream of the possible ORF-1 (putative mob). Two putative promoter structures at the -35 region (TTTACT; np 1341–1346) and -10 region (TTATCA; np 1359–1364), as well as the start codons for a possible ORF-2 (np 1378–1380), for ORF-3 (np 1511–1513) and for ORF-4 (np 2553–2555) were also identified as transcriptional promoters immediately upstream of the possible ORF-2 for ORF-2, -3 and -4. However, whether or not these promoter sequences are functional is unclear, and expression experiments are required.

Probable ribosome-binding (RB) sites (Shine-Dalgano [SD] sequences),[32,33] which are complementary to a highly conserved sequence of CCUCCU close to the 3′ end of 16S ribosomal RNA (AAGA [np 3198–3201] for the possible ORF-1 [mob]; AGG [np 1369–1371] for ORF-2 [repA]; and GGA [np 1507–1509] for ORF-3 [repB]) were also identified.

Discussion

Prior to starting the present study, the authors screened for the presence of plasmid DNA in a total of 47 UPTC isolates from the natural environment, including wild birds in Northern Ireland, England, Japan, and from humans in France. The results indicated the presence of plasmid DNA in 12 out of the 47 isolates, at a frequency of approximately 26%.[29] This value is similar to the approximate 33% frequency for *C. jejuni* and *C. coli*.[34]

The demonstration of high sequence similarities of 58.2% and 56.4% of pUPTC237 whole DNA with pCL300[28] and with pCJ419 DNA,[25] respectively, may suggest a shared ancestry among the three plasmids.

A hypothetically intrinsic transcription terminator structure, which contains a G+C-rich region near the base of the stem (np 2900–2926) and a single-strand run of U (np 2927–2930) residues, was demonstrated downstream of the ORF-4, as shown in Figure 3. Thus, ORF-1, -2, -3 and -4 may all constitute an operon and may generate a messenger RNA (mRNA) transcript.

Northern blot hybridisation of the total RNA purified from the UPTC237 isolate cells containing pUPTC237 plasmid with the *mob* and *repB* fragments amplified as probes was performed in order to clarify the expression of the ORFs. As shown in Figure 4, an RNA transcript was hybridised with *mob* and *repB* fragment probes. In addition, no hybridisation signals were detected for the total RNA of the UPTC2 and UPTC476 isolate cells, which contained no plasmid DNAs.□

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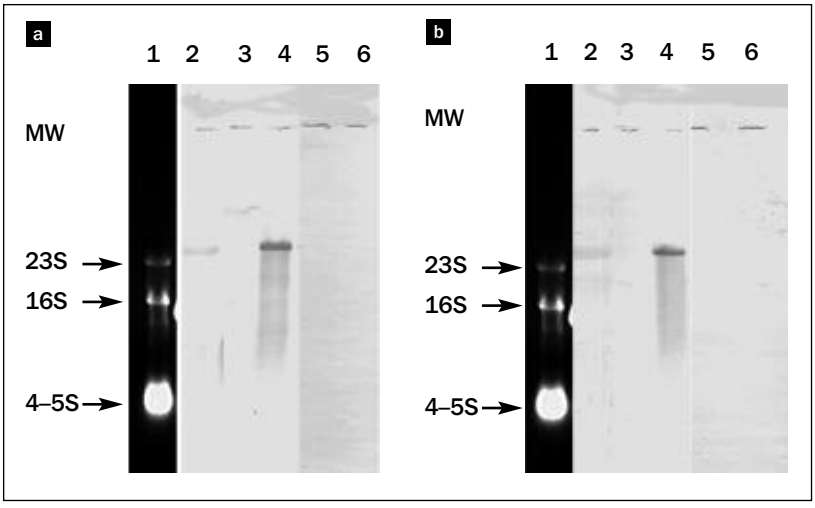

Fig. 4. An RNA transcript (approximately 3000 bp, Lane 2) hybridised with (a) *mob* and (b) *repB* fragment probes. Lane 3: RNase-digested total RNA fraction of UPTC237. Lane 4: pUPTC237 DNA. No hybridisation signals were detected for the total RNA of the UPTC2 (Lane 5) and UPTC476 (Lane 6) isolate cells.

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