Uneven distribution of the *luxS* gene within the genus *Campylobacter*

A TAZUMI[*], M NEGORO[*], Y TOMIYAMA[*], N MISAWA[†], K ITOH[‡], J E. MOORE[§#], B. C MILLAR[#] and M. MATSUDA[*]

[*]Laboratory of Molecular Biology, School of Environmental Health Sciences, Azabu University, Sagamihara; [†]Department of Veterinary Public Health, Faculty of Agriculture, Miyazaki University, Miyazaki; [‡]Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Japan; [§]School of Biomedical Sciences, University of Ulster, Coleraine; and [#]Department of Bacteriology, Northern Ireland Public Health Laboratory, Belfast City Hospital, Belfast, Northern. Ireland, UK

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ABSTRACT

Polymerase chain reaction (PCR) amplification was performed on 20 isolates of five *Campylobacter* species using a degenerate primer pair designed *in silico* to generate a product of the *luxS* gene or its homologue from *Campylobacter* organisms. Although the primer pair successfully amplified products of approximately 500 base pairs (bp) with the eight isolates of *C. jejuni* and *C. coli* and some of *C. upsaliensis* and *C. fetus*, it failed to amplify fragments with all four isolates of *C. lari* (two urease-negative *C. lari*; two urease-positive thermophilic campylobacters). When Southern blot hybridisation analysis was carried using the mixed *luxS* gene fragments prepared from the *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* strains as a probe, all *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* isolates gave positive signals, but no positive signal was detected with any *C. lari* isolate. These results clearly indicate that *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* carry the *luxS* gene or its homologue. However, no *luxS* gene or its homologue was identified to occur in the *C. lari* genome. Although autoinducer-2 assays were positive in *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* isolates, it was negative with all the *C. lari* isolates examined. In addition, a biofilm formation assay demonstrated that biofilm formation in the *C. lari* species does not appear to correlate with the occurrence of the *luxS* gene because biofilm formation occurred among some isolates of *C. lari*.

KEY WORDS: Autoinducer-2.
Biofilms.
Campylobacter.
LuxS protein, bacteria.

Introduction

Bacterial cell-to-cell communication is referred to as quorum sensing,[1] a population-dependent signalling mechanism that involves the production and detection of extracellular signalling molecules.[2] Acyl-homoserine lactones produced in Gram-negative bacteria are classified as autoinducer-1 (AI-1),[1] and an alternative quorum sensing mechanism existing in Gram-negative and Gram-positive bacteria is mediated by a furanosyl borate diester,[3] referred to as AI-2. [4] The *luxS* gene is responsible for AI-2 production (AI-2 synthase).[1]

Thermophilic *Campylobacter* species, primarily *C. jejuni* and *C. coli*, are curved Gram-negative bacteria that are the recognised cause of campylobacteriosis worldwide.[6] In relation to human campylobacteriosis, *C. lari, C. upsaliensis* and *C. fetus* have also been demonstrated to be implicated as gastrointestinal pathogens, although some are rare.[7]

In relation to quorum sensing in *Campylobacter* organisms, it has been reported that *C. jejuni* and *C. coli* possess the *luxS* gene, required for AI-2 production in other bacterial species.[2,8] Joen *et al.* also reported that *C. jejuni* 81116 isogenic *luxS*-null mutant (23281) reduced transcription of the major flagellin gene, *flaA* (approximately 43% that of the wild-type),[9] and the *cdt* genes (*cdtA, cdtB* and *cdtC*) encoding the cytolethal distending toxin (CDT; approximately 61%).[10] Most recently, Miller *et al*. reported the *luxS* gene to be absent from the *C. lari* RM2100 genome, following studies on the completed genomic sequence of the human clinical isolate RM2100.[11] However, to the authors' knowledge, there has been no structural analysis of the *luxS* gene or its homologues from *Campylobacter* organisms other than *C. jejuni* and *C. coli.*

The aim of the present study is to construct a PCR primer pair *in silico* for amplification of the full-length *luxS* structural gene segment and then to attempt to amplify the *luxS* gene using 20 isolates of five *Campylobacter* species including the major and typical species *C. jejuni, C. coli* and *C. fetus*, as well as atypical *C. lari*, including urease-negative (UN) *C. lari* and urease-positive thermophilic *Campylobacter* (UPTC)[12,13] and *C. upsaliensis*. Furthermore, the authors wish to clarify whether or not the *luxS* gene or its homologues occur in 20 isolates by Southern blot hybridisation analysis. AI-2 production assays and biofilm formation will also be examined.

Correspondence to: Professor Motoo Matsuda
Laboratory of Molecular Biology, Graduate School of Environmental Health Sciences, Azabu University, Fuchinobe 1-17-71, Sagamihara 229-8501, Japan
Email: matsuda@azabu-u.ac.jp

Materials and methods

The 20 isolates of five *Campylobacter* species analysed in the present study are shown in Table 1. These isolates were cultured on Mueller-Hinton agar (Oxoid, Hampshire, UK) containing 5% (v/v) defibrinated horse blood (Nippon Bio-

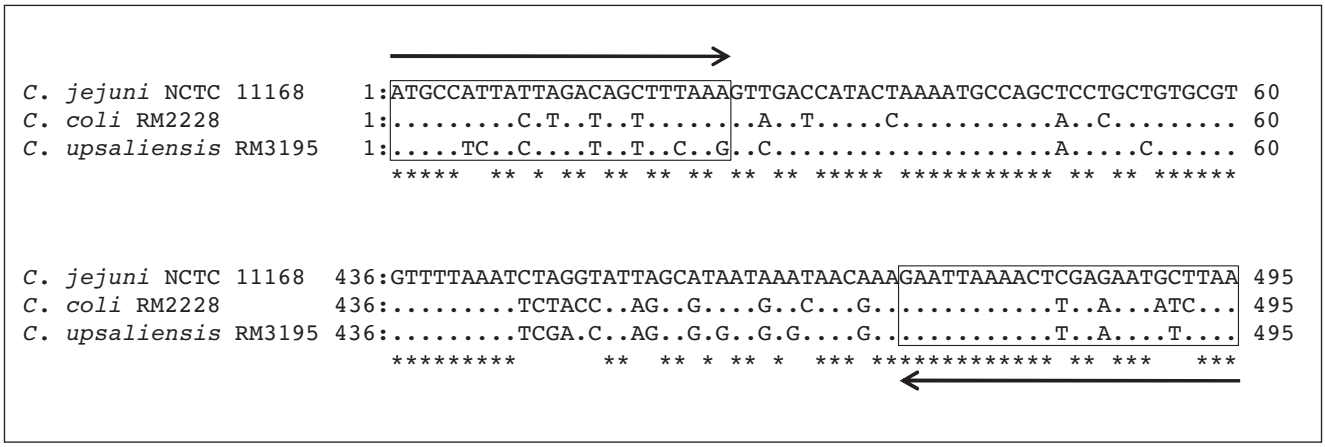

Fig. 1. Nucleotide sequence alignment analysis to design a degenerate PCR primer pair (f-/r-CluxS) for amplification of the full-length *luxS* structural gene from campylobacters. Nucleotide sequences of the *lux*S structural gene from *C. jejuni* NCTC11168, *C. coli* RM2228 and *C. upsaliensis* RM3195 were aligned. Dots indicate identical bases; changes are indicated. Numbers at the left and right refer to base pairs of the full-length *lux*S structural genes from the three isolates, respectively.

Test, Tokyo, Japan) at 37°C for 48 h in an aerobic jar under microaerophilic conditions.

Genomic DNA was prepared from the cells using sodium dodecyl sulphate, proteinase K and cetyltrimethylammonium bromide treatment and phenol-chloroform extraction and ethanol precipitation.[14] A degenerate polymerase chain reaction (PCR) primer pair (f-CluxS: 5′-ATGCCWYTAYTWGAYAGYTTYAAR-3′; r-CluxS: 5′-TTARRYATTYTCRAGTTTTAATTC-3′) was designed *in silico* for amplification of the full-length *luxS* structural gene, based on sequence information of the *luxS* structural gene from *C. jejuni* NCTC11168 (DDBJ/EMBL/GenBank Accession No. AL111168), *C. coli* RM2228 (AAFL01000002) and *C. upsaliensis* RM3195 (AAFJ01000005). This primer pair was expected to generate a product of the *luxS* gene segment of approximately 500 bp with *Campylobacter* organisms. The PCR method was performed in 25 µL reaction volumes at 94°C for 5 min, then 30 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec, followed by a final extension of 72°C for 7 min. Amplified products were then separated and purified, as previously described.[15] Following purification of all the amplicons of the *luxS* gene, nucleotide sequencing and sequence analysis were carried out, as previously described.[15]

Southern blot hybridisation analysis for the *luxS* gene segment was carried out using digoxigenin (DIG)-labelled *luxS* gene PCR fragments amplified and prepared as probes with *Hin*dIII-digested whole genome DNAs from isolates of the five *Campylobacter* species according to the procedure described by Sambrook and Russell.[14] The PCR fragments for the mixed probes were amplified using a primer pair of f-/r-CluxS with the *C. jejuni* LMG6444, *C. coli* NCTC11366, *C. upsaliensis* 104-1 and *C. fetus* cf2-1 strain DNA templates. Random primer extension was performed in order to prepare the mixed probes using DIG High Prime (Roche Diagnostic, Penzberg, Germany).

The AI-2 assay was performed as described by Surette *et al*.[5] and Jeon *et al*.[9] *Campylobacter* isolates were cultured on Mueller-Hinton agar plates containing defibrinated horse blood (5% v/v; Nippon Bio-Test) under microaerophilic conditions (5% [v/v] O_2, 10% [v/v] CO_2 and 10% [v/v] H_2) at 37°C for 48 h. Cells were then inoculated in Mueller-Hinton broth with initial absorbance (A_{600}) of approximately 0.4. Cells were inoculated at a 1 in 200 dilution and incubated in 24-well polystyrene plates (Corning, Tokyo, Japan). Preparation of cell-free supernatant (CFS) from *Campylobacter* cells was carried out by filtration through a 0.22 µm Millex syringe-driven filter unit (Millipore, Bedford, MA, USA). The prepared CFS was immediately stored at –30°C until used.

Vibrio harveyi BB170 was grown with aeration in Nutrient Broth No. 2 at 30°C for 16 h, inoculated on AB medium and grown at 30°C for 16 h. The diluted culture of *V. harveyi* BB170 (180 µL; at 1 in 5000) was mixed with 20 µL CFS of the tested isolates in sterile glass tubes. Sterile medium was used as a negative control and CFS of *C. jejuni* 81116 was used as a positive control. The tubes were incubated in a water bath at 30°C with shaking for 5 h. A portion (100 µL) of each mixture was collected to measure bioluminescence production (Luminescencer-JNR AB-2100; ATTO Corp., Tokyo, Japan). Each sample was prepared in three different

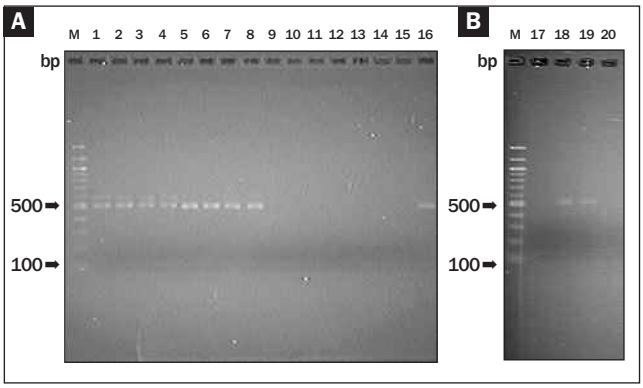

Fig. 2. Polymerase chain reaction profiles of the full-length *luxS* structural gene amplified from 20 isolates of *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis* and *C. fetus* using f-/r-CluxS primer pair. M: 100 bp DNA ladder. A) Lane 1: *C. jejuni* LMG6444; lane 2: 81-176; lane 3: HP5090; lane 4: HP5122; lane 5: *C. coli* NCTC11366; lane 6: 23; lane 7: 165; lane 8: JCM2529^T; lane 9: *C. lari* JCM2530^T; lane 10: 84C-1; lane 11: UPTC NCTC12893; lane 12: UPTC 89049; lane 13: *C. upsaliensis* G1104; lane 14: 12-1; lane 15: 60-1; lane 16: 104-1. B) Lane 17: *C. fetus*, ATCC27374; lane 18: cf2-1; lane 19: 8414c; lane 20: 8215a.

Table 1. Isolates of five *Campylobacter* species, biofilm formation and AI-2 production analysed in the present study.

Organism	Isolate	Source	Note	AI-2 production	Biofilm formation (A570)
C. jejuni	81116	Human	NCTC11828	3610	0.974
C. jejuni	LMG6444	Human	Our collection	5613	1.019
C. jejuni	81-176	Human	Our collection	5774	0.400
C. jejuni	HP5090	Human	Our collection	5892	0.059
C. jejuni	HP5122	Human	Our collection	6018	0.773
C. coli	NCTC11366	Pig	NCTC	2849	0.377
C. coli	23	Dog	Our collection	2424	0.410
C. coli	165	Seagull	Our collection	2060	0.484
C. coli	JCM2529^T	Pig	Our collection	2549	0.125
C. lari (UN *C. lari*)	JCM2530^T	Seagull	JCM	67	0.644
C. lari (UN *C. lari*)	84C-1	Human	Our collection	100	0.312
C. lari (UPTC)	NCTC12893	River water	NCTC	667	0.313
C. lari (UPTC)	89049	Human	F. Megraud	343	0.782
C. upsaliensis	G1104	Dog	Our collection	1328	0.023
C. upsaliensis	12-1	Dog	Our collection	2343	0.776
C. upsaliensis	60-1	Dog	Our collection	3480	0.806
C. upsaliensis	104-1	Cat	Our collection	2007	0.642
C. fetus	ATCC27374	Sheep	Our collection	4032	0.155
C. fetus	cf2-1	Bovine (stool)	Our collection	3323	0.431
C. fetus	8414c	Bovine (bile)	Our collection	4585	0.515
C. fetus	8215a	Bovine (bile)	Our collection	3285	0.265

UN *C. lari*, urease-negative *C. lari*; UPTC, urease-positive thermophilic *Campylobacter*; F. Megraud, Insern U853, France; AI, autoinducer; A_{570}: absorbance at 570 nm.

tubes. AI-2 production was examined using Light Capture AE-6961/C/FC (ATTO Corp.), where a value of 1000 or less was regarded as negative.

A biofilm assay to determine biofilm formation was carried out according to a procedure described previously.[16] *Campylobacter* cell culture was carried out as described for the AI-2 assay. Following culture, the medium was removed, the wells were dried at 55°C for 30 min and 0.1% crystal violet (CV) was added at room temperature for 5 min. The wells were washed twice with H_2O and dried at 55°C for 15 min. Following addition of 100% ethanol, absorbance at 570 nm (A_{570}) was determined using a GeneQuant 1300 Spectrophotometer (GE Healthcare, England, UK) to determine biofilm formation. Using CV staining, biofilm formation was negative when absorbance values were ≤0.1 (at 570 nm).

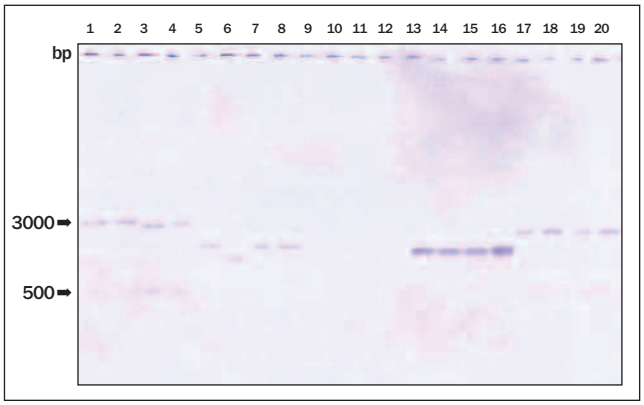

Fig. 3. Southern blot hybridisation analysis of genomic DNA digested with *Hin*dIII from 20 isolates of five *Campylobacter* species using the *luxS* gene fragment as a probe. See the legend to Figure 2 for lanes 1–20.

Results and discussion

The PCR amplification experiments were first carried out with 20 isolates of five *Campylobacter* species (*C. jejuni, C. coli, C. lari, C. upsaliensis* and *C. fetus*) using a degenerate primer pair (f-/r-CluxS) which was designed to generate a product of the full-length *luxS* structural gene from *Campylobacter* organisms (Fig. 1). Some of the PCR amplification profiles are shown in Figure 2. The primer pair successfully amplified products of approximately 500 bp with the eight isolates of *C. jejuni* and *C. coli* and some *C. upsaliensis* and *C. fetus*. isolates. However, the primer pair failed to amplify fragments with all four isolates of *C. lari* (lanes 9-12, Fig. 2). Consequently, the primer target regions of the *luxS* gene of some isolates of *Campylobacter* examined may be not completely conserved, not allowing hybridisation to the primers. Alternatively, these *luxS* PCR-negative isolates may not harbour the *luxS* gene or its homologue in their genomes, as no product was generated reproducibly with these isolates using the primer pair f-/r-CluxS.

Therefore, in order to clarify this, an attempt was made to perform Southern blot hybridisation using DIG-labelled *luxS* gene PCR fragment mixtures amplified and prepared as

probes. Although, as shown in Figure 3, *Hin*dIII-digested whole genome DNAs prepared from all 16 isolates of *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* gave positive hybridisation signals, no signals were detected with all four isolates of *C. lari*. These results indicate that *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* carry the *luxS* gene or its homologue in their genomes, whereas *C. lari* does not appear to do so.

Regarding *C. lari* organisms, Southern blot hybridisation analysis was performed on the other 30 isolates (15 UN *C. lari*, 15 UPTC). However, no positive signals were detected (data not shown). Therefore, these *C. lari* organisms were confirmed not to carry *luxS* genes or their homologues.

The *luxS* gene is highly conserved in a wide range of Gram-positive and Gram-negative bacteria,[17] and synthesis of AI-2 depends on the *luxS* gene. Therefore, it would be worthwhile to know if AI-2 synthesis occurred in the *Campylobacter* organisms of the five species analysed in the present study. AI-2 synthesis was then examined among the 20 *Campylobacter* isolates using a bioluminescence production assay. *C. jejuni* 81116 produces AI-2,[9] as shown in Table 1. Therefore, the AI-2 produced by *C. jejuni* 81116 was employed as a positive control. All 16 isolates of the four *C. jejuni, C. coli, C. upsaliensis* and *C. fetus* species produced AI-2 (Table 1). However, no AI-2 production was identified in all four *C. lari* isolates (Table 1).

It is suggested that AI-2 is a universal bacterial quorum-sensing signal molecule synthesised by the LuxS enzyme, which is involved in the AI-2 synthesis pathway both in Gram-negative and Gram-positive bacteria. Biofilm formation by bacterial cells is thought to be under the control of AI molecules. In addition, both positive and negative correlations between LuxS and biofilm formation have been described in many bacteria.[18]

As shown in Table 1, biofilm formation occurred in some of the isolates (*C. jejuni* LMG6444, HP5122, *C. coli* 165, *C. upsaliensis* 12-1, 60-1, 104-1 and *C. fetus* cf2-1 and 8414c) but not in others (*C. jejuni* 81-176, HP5090, *C. coli* NCTC11366, 23, JCM2529^T, *C. upsaliensis* G1104 and *C. fetus* ATCC27374), although some variation in the ability to form biofilm was observed among the isolates examined. Moreover, biofilm formation on abiotic surfaces was also identified in some of the isolates of *C. lari* (*C. lari* JCM2530^T and UPTC 89049) in which no positive signals were detected by Southern blot hybridisation. Some isolates of *Campylobacter* including *C. lari* were biofilm-negative following the addition of CFS containing the AI-2 from *C. jejuni* 81116 and *C. lari* UPTC 89049, both of which are positive for AI-2 production. Consequently, biofilm formation in the *C. lari* species, as well as other campylobacters, does not appear to correlate with the presence of the *luxS* gene.

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