Quorum sensing in *Campylobacter jejuni*: detection of a *luxS* encoded signalling molecule

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The expression of a wide variety of physiological functions in many bacterial species is modulated by quorum sensing, a population-dependent signalling mechanism that involves the production and detection of extracellular signalling molecules. The genome sequence of *Campylobacter jejuni* NCTC 11168 contains a gene encoding an orthologue of LuxS, which is required for autoinducer-2 (AI-2) production in other bacterial species, but does not contain genes predicted to encode any known acyl-homoserine lactone synthetase. This study demonstrates that *C. jejuni* produces functional AI-2 activity through the ability of cell-free extracts to specifically induce bioluminescence in *Vibrio harveyi* BB170, a reporter strain for quorum-sensing system 2. Production of this signalling compound was shown to be dependent upon the product of the *C. jejuni luxS* gene (Cj1198). While the *luxS* mutant showed comparable growth rate, resistance to oxidative stress and ability to invade Caco-2 cell monolayers to the parental strain, it exhibited decreased motility haloes in semisolid media, suggesting a role for quorum sensing in the regulation of motility.

Keywords: LuxS, autoinducer-2, campylobacters

INTRODUCTION

Campylobacter jejuni is the most common bacterial cause of human gastrointestinal infection worldwide, and in many countries the incidence of infection continues to increase. In England and Wales, for example, there were almost 54 000 reported cases during 2000 and the number of cases of gastroenteritis attributed to *Campylobacter* is now three and a half times more than those due to *Salmonella* (Anonymous, 2001). The main symptoms of this food-borne disease are malaise, fever, severe abdominal pain and diarrhoea. Although the acute phase is usually self-limiting, it often persists for up to a week with abdominal pain continuing for many more days. Serious sequelae include acute neuromuscular paralysis due to Guillain-Barré syndrome, which is estimated to affect one in every thousand campylobacteriosis cases (Nachamkin *et al.*, 1998).

C. jejuni is associated with many domesticated animals

and birds, and consequently four common sources, poultry, raw milk, water and pets, account for nearly all cases of human infection. Although the ability of campylobacters to survive in food and the environment is primarily important to their infective and contamination cycle, little is known of the adaptive survival responses that these organisms are able to initiate in response to exposure to environmental stress. Information contained within the recently completed genome sequence for *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000), however, has provided some important insights into the physiology of this pathogen. Most notably, it has become apparent that, whilst *C. jejuni* can survive in a diverse range of environments, its capacity for regulating gene expression in response to environmental stress appears to be very limited compared to other bacteria, including *Escherichia coli* and *Bacillus subtilis* (Park, 2000).

Bacteria differ in their response to environmental stress, but all have at least some capacity for monitoring the environment for changes, which require an adaptive response in order to survive. In the past decade, it has become apparent that many bacterial species are able to

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Abbreviations: AI-1/2, autoinducer-1/2; HSL, homoserine lactone.

regulate a wide variety of traits in response to cell density. Cell-cell communication, or quorum sensing, involves the synthesis, secretion and detection of extra-cellular signalling molecules termed autoinducers (Bassler, 1999). When these molecules reach a critical threshold concentration within a population, a signal transduction cascade is triggered, which forms the basis for alterations in gene expression.

Quorum sensing in many Gram-negative bacteria is based upon the signalling molecule homoserine lactone (HSL) which controls the expression of numerous traits including bioluminescence, antibiotic production and virulence factor production (Fuqua & Greenberg, 1998; De Kievit & Iglewski, 2000; Bassler, 1999). In *Pseudomonas aeruginosa*, for example, two HSLs control the expression of a number of extracellular virulence factors (Pearson *et al.*, 1994) and are also involved in biofilm differentiation (Davies *et al.*, 1998). *P. aeruginosa* has also been shown to produce two other types of signalling molecule, a quinolone signalling molecule that regulates the expression of the virulence gene *lasB* (Pesci *et al.*, 1999; McKnight *et al.*, 2000) and diketopiperazines, which at high concentrations are able to cross-activate HSL LuxR-based quorum-sensing systems (Holden *et al.*, 1999). Gram-positive bacteria generally secrete processed peptide signalling molecules, which act via a membrane-bound histidine protein kinase (Lazazzera & Grossman, 1998; De Kievit & Iglewski, 2000; Bassler, 1999; Mayville *et al.*, 1999). In *Vibrio harveyi*, another quorum-sensing system has recently been identified that produces the signalling molecule autoinducer-2 (AI-2). This system is highly conserved in both Gram-positive (Kuroda *et al.*, 2001) and Gram-negative bacteria and thought to be used for interspecies communication (Bassler, 1999). The chemical structure of the cognate signalling molecule AI-2 has recently been predicted as a furanone (Schauder *et al.*, 2001) and the *luxS* gene codes the final enzyme in the biosynthetic pathway for its production. AI-2 is detected by a sensory histidine kinase located within the cytoplasmic membrane (Surette *et al.*, 1999).

Several species, including *E. coli* (Sperandio *et al.*, 1999), *Salmonella typhimurium* (Surette & Bassler, 1999), *Shigella flexneri* (Day & Maurelli, 2001), *Helicobacter pylori* (Forsyth & Cover, 2000; Joyce *et al.*, 2000) and *Vibrio vulnificus* (McDougald *et al.*, 2001) have been shown to produce an AI-2-like activity. This study demonstrates *luxS*-dependent production of an extra-cellular signalling molecule by *C. jejuni* with AI-2-like activity.

METHODS

Strains and culture conditions. *C. jejuni* NCTC 11168 was obtained from the National Collection of Type Cultures (PHLS). The *V. harveyi* reporter strain BB170, capable of sensing AI-2 but not AI-1, and *V. harveyi* BB152, capable of producing AI-2 but not AI-1, were both generous gifts from B. Bassler (Princeton University, USA). *E. coli* DH5 α was obtained from Life Technologies. *C. jejuni* NCTC 11168 was

cultured at 37 °C under microaerobic conditions using the CampyGen system (Oxoid) on Mueller-Hinton (M-H) agar (Oxoid). *V. harveyi* strains were grown at 30 °C on Luria-Marine agar plates (Bassler *et al.*, 1994) or in Autoinducer Bioassay (AB) medium (Greenberg *et al.*, 1979). *E. coli* DH5 α was grown at 37 °C in Luria-Bertani (LB) broth. Ampicillin and kanamycin were included in the growth media at 50 $\mu\text{g ml}^{-1}$ for plasmid maintenance when required.

Generation of *C. jejuni luxS* mutant. Genomic DNA was isolated from *C. jejuni* using guanidium thiocyanate (Pitcher *et al.*, 1989). The putative *luxS* from *C. jejuni* NCTC 11168 (accession no. AL111168; Parkhill *et al.*, 2000) was amplified from genomic DNA using PCR and the oligonucleotide primers LUX1 5'-AGGCAAAGCTCCTGGTAAGGCCA-A-3' and LUX2 5'-GGATCCGTATAGGTAAGTTCAT-TTTTGCTCC-3'. The PCR reaction contained 0.5 μmol each primer, *Taq* DNA polymerase (Qiagen) and dNTPs (Roche; 200 μmol). PCR reactions were performed using a Perkin-Elmer GeneAmp PCR system 2400 thermal cycler. Following an initial denaturation for 3 min at 94 °C, there were 30 cycles of amplification comprising 30 s denaturation at 94 °C, 60 s annealing at 55 °C and a 60 s extension at 72 °C. There was a final extension for 10 min at 72 °C. The 1080 bp DNA fragment generated in this manner was cloned into pBAD-TOPO TA cloning vector (Invitrogen) and transformed into *E. coli* DH5 α . The resulting plasmid was designated pKE25 and in this vector the putative *luxS* gene was orientated so that its transcription could be initiated from the inducible *araB* promoter.

The *luxS* gene was mutated using the inverse PCR protocol described by Wren *et al.* (1993). This was used to introduce a unique *Bgl*II restriction site and to delete 50 bp of the gene. Oligonucleotide primers LUX3 5'-caacaAGATCTCCCG-TGCGACAACCCATAGGTGAA-3' and LUX4 5'-caacaAGATCTCTTGGAAGCAGCCATGAAAGATG-3' with unique restriction sites for *Bgl*II (underlined) and clamp sequences, not complementary to *C. jejuni* DNA (lower case) were used. In this PCR, a two-stage cycling programme was used to amplify longer DNA fragments. The first stage comprised 10 cycles, each including 10 s denaturation at 94 °C, 60 s annealing at 58 °C and a 5 min 15 s extension at 68 °C. The following stage comprised a further 25 cycles, each including 10 s denaturation at 94 °C, 60 s annealing at 58 °C and a 5 min 15 s extension at 68 °C with 10 s added for each cycle number. The resulting PCR fragment was digested with *Bgl*II, self-ligated and transformed into *E. coli* DH5 α . The resulting plasmid, pKE27, was next digested with *Bgl*II and a kanamycin resistance cassette with *Bam*HI ends, from pJMK30 (J. Ketley, University of Leicester), inserted into this site. This step generated the suicide plasmid pKE28, which was introduced into *C. jejuni* NCTC 11168 competent cells by electroporation at 2.5 kV, 200 Ω and 25 μF . Three *luxS* mutants generated by a double homologous recombination event were designated CJLUXS01-03.

DNA-DNA hybridizations. These were carried out using a non-radioactive AlkPhos direct labelling and detection system (Amersham Pharmacia Biotech). Chromosomal DNA was isolated from kanamycin-resistant transformants and wild-type cells, and digested with the restriction endonuclease *Xmn*I. The resulting fragments were separated on a 0.8% agarose gel. The DNA was depurinated (0.125 M HCl), denatured (1.5 M sodium chloride/0.5 M sodium hydroxide) and transferred to Hybond-N+ nitrocellulose membranes using an alkali transfer procedure (Amersham Pharmacia Biotech). A probe generated by PCR using primers LUX1 and

LUX2 with genomic DNA as template was labelled with thermostable alkaline phosphatase and used to detect homology according to the manufacturer's instructions.

Preparation of cell-free culture medium for AI-2 assays. Cells of *C. jejuni* were grown to confluence on M-H agar. After 16 h growth, the cultures were harvested in 1 ml AB medium per plate and the OD₆₀₀ of each cell suspension was adjusted to 0.4 with AB medium using a Helios Alpha spectrophotometer (Unicam) and cuvettes with a 1 cm path length. Cell-free culture medium was prepared from these suspensions by centrifugation for 15 min at 8000 g, followed by filtration of the supernatant through a Minisart 0.2 µm single-use filter unit (Sartorius). Cell-free culture media from *V. harveyi* strains, *E. coli* DH5α containing pBAD-TOPO, and *E. coli* DH5α expressing the *C. jejuni luxS* gene were prepared in the same manner, except that the cells were cultured aerobically in AB medium at 30 °C and 37 °C respectively.

AI-2 bioluminescence assay. The AI-2 reporter strain, *V. harveyi* BB170, was grown for 16 h with aeration (175 r.p.m.) at 30 °C in AB medium, and then diluted 1:5000 in fresh AB medium. Cell-free preparations were then added to the diluted *V. harveyi* culture at a 10% (v/v) final concentration. The flasks were incubated at 30 °C with aeration (175 r.p.m.) and aliquots of 100 µl were removed hourly for total luminescence measurements using Lumac/3M biocounter 2010A. Growth of the reporter strain was also measured by OD₆₀₀ and by viable cell counts with appropriate dilution.

Growth and survival of *C. jejuni*. Bacterial suspensions of *C. jejuni* NCTC 11168 and CJLUXS01 were prepared in M-H broth from confluent lawns and adjusted to an OD₆₀₀ of 0.3. Flasks containing 50 ml M-H broth were inoculated with 100 µl cell suspension and incubated at 37 °C microaerobically and aerobically at 125 and 250 r.p.m., respectively. Growth and survival of the cultures was monitored by viable counts.

Motility assays and resistance to oxidative stress. Motility assays were performed at 37 °C on 0.4% agar plates containing M-H broth. The motility haloes were measured after 24 h. Growth inhibition of *C. jejuni* NCTC 11168 and CJLUXS01 due to paraquat (Sigma) and hydrogen peroxide (Sigma) was monitored using well diffusion susceptibility assays. Cell suspensions (100 µl) were spread onto M-H plates and 6 mm wells cut into the agar were filled with 30 µl of different concentrations of paraquat (1–40 mM) and hydrogen peroxide (0.3–0.015%, v/v). Plates were incubated microaerobically at 37 °C for 24 h. Zones of inhibition were then measured.

Production of *C. jejuni* signalling molecule at different growth phases. To examine the kinetics of autoinducer production in *C. jejuni* NCTC 11168 and CJLUXS01, cell-free culture media were prepared at time points reflecting all phases of growth from broth cultures. Cultures were grown microaerobically at 37 °C at 125 r.p.m. At 5, 18, 28 and 42 h, 4 ml samples were removed from the flasks and used to prepare cell-free culture media (described above) and assessed for viable counts. The AI-2 bioassay was carried out as described above.

Mammalian cell culture. Stock cultures of Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC), Porton Down, UK. They were grown as monolayers in Minimal Essential Medium Eagle (MEM; Sigma) with 10% (v/v) foetal bovine serum (Gibco-BRL), 1% (v/v) 200 mM glutamine (Gibco-BRL) and 1% (v/v) non-essential amino acids (Sigma). No antibiotics were added. The cultures

were incubated in a CO₂ 5% (v/v) incubator at 37 °C. For experimental assays, cultures were harvested by trypsinization and seeded into 6-well tissue culture trays at 3.8×10^5 cells per well and incubated 24 h in a CO₂ incubator at 37 °C.

Adherence and invasion assay. Bacterial suspensions of *C. jejuni* NCTC 11168 and CJLUXS01 were prepared in M-H broth from confluent plates and adjusted to OD₆₀₀ 0.3. The suspensions were centrifuged at 3000 r.p.m. for 15 min at 15 °C. The pellet was resuspended in MEM and viable counts were carried out on M-H agar. Caco-2 cell cultures in 6-well plates were inoculated with 7.8×10^8 c.f.u. *C. jejuni* NCTC 11168 or CJLUXS01 in 0.5 ml; 12 wells in total were inoculated for each strain. The plates were incubated at 37 °C in 5% CO₂ for 3 h to allow the bacteria to adhere to and invade the epithelial cells. The monolayers were washed three times with pre-warmed Hanks Balanced Salt Solution (HBSS; Sigma) and then six of the wells for each strain were incubated for 1.5 h in 2 ml pre-warmed MEM with 100 µg gentamicin sulphate ml⁻¹ (Sigma). In the remaining wells only MEM (2 ml) was added. *C. jejuni* NCTC 11168 and CJLUXS01 were shown to be sensitive to gentamicin with no viable cells recovered after 15 min exposure to the antibiotic (data not shown). The monolayers were washed three times with HBSS and lysed with 0.1% (v/v) Triton X-100 in PBS (Sigma). The lysed monolayer suspensions were serially diluted and spread on M-H agar for viable counts of *C. jejuni*.

Statistical analysis. Comparisons between *C. jejuni* NCTC 11168 and CJLUXS01 in adherence and invasion of Caco-2 cells and the motility assays were analysed using the unpaired Mann–Whitney two sample test using Minitab. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

Identification of AI-2 activity in *C. jejuni*

The genome sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000) was analysed for the presence of genes encoding acyl-homoserine lactone synthetases and LuxS orthologues which may be required for autoinducer production. Whilst the genome sequence did not contain genes predicted to encode any known acyl-homoserine lactone synthetase, the analysis revealed the presence of a hypothetical protein (Cj1198), which had 74% amino acid identity to LuxS from *V. harveyi*. Cj1198 also showed 71% identity to LuxS from *E. coli*, 70% identity to LuxS from *Haemophilus influenzae*, 73% identity to LuxS from *Pasteurella multocida* and 40% amino acid identity to LuxS from *Helicobacter pylori* (Fig. 1).

To test whether *C. jejuni* NCTC 11168 produces a signalling molecule related to AI-2, a bioassay was carried out using the *V. harveyi* reporter strain BB170. In this strain, which does not have a functional sensor for AI-1, but has an intact sensor for AI-2, the expression of bioluminescence can be correlated with the presence of AI-2 alone. *V. harveyi* BB152, which is capable of producing AI-2 but not AI-1, was used as the positive control for AI-2 activity. In the absence of a heterologous source of AI-2, demonstrated here by the control experiment, which used unconditioned AB medium, the bioluminescence generated by the *V. harveyi* reporter strain BB170 steadily declined until the 4 h time point

<i>C. jejuni</i>	MPL---LDSEKVDHTKMPAPAVRLAKVMKTPKGGDISVFDLRF CIP NKDIMSEKGTHTL	56
<i>V. harveyi</i>	MPL---LDSEFTVDHTRMNA PAVR VAKTMTQTPKGDITVFDLRF TAP NKDILSEKGIHTL	56
<i>E. coli</i>	MPL---LDSEFTVDHTRMEAPAVRVAKTMTTPHGDAITVFDLRF CV PNKEVMPERGIHTL	56
<i>H. influenzae</i>	MPL---LDSEKVDHTKMNAPAVRIAKTMTTPKGDNITVFDLRF CIP NKEILSPKGIHTL	56
<i>P. multocida</i>	MPL---LDSEKVDHTKMPAPAVRIAKTMTTPKGDNITVFDLRF CIP NKEILSPKGIHTL	56
<i>C. perfringens</i>	MVK---VESFELDHTKVKAPYVRKAGIKIGPKGDIVSKFDLRF VQ PNKELLSDKGMHTL	56
<i>B. subtilis</i>	MPS---VESFELDHNNAVAPYVRHC GVH KVGTG GVN KEDIRF CQ PNQAMKPDITHTL	56
<i>H. pylori</i>	MKTPKMNVESFNLDHTKVKAPYVRVADRKKGVNGDLIVKYDVRF KQ PNQDHMDMPSLSHL	60
<i>C. jejuni</i>	EHFLAGFMRDHLNS-NSVEIIDISPMGCR TGF YMSLIGTPDEKSIKAWAAMKDVLSVS	115
<i>V. harveyi</i>	EHLYAGFMRNHLNG-DSVEIIDISPMGCR TGF YMSLIGTPSEQVADAWIAAMEDVLKVE	115
<i>E. coli</i>	EHFLAGFMRNHLNG-NGVEIIDISPMGCR TGF YMSLIGTPDEQVADAWKAAMEDVLKVQ	115
<i>H. influenzae</i>	EHFLAGFMRNHLNG-DSIEIIDISPMGCR TGF YMSLIGTPNEQKVSEAWLASMQDVLGVQ	115
<i>P. multocida</i>	EHFLAGFMRDHLNG-TEVEIIDISPMGCR TGF YMSLIGTPNEQKVSEAWLASMQDVLGVQ	115
<i>C. perfringens</i>	EHFLAGFMRKLD----DVIDISPMGCR TGF YLSFGDIDVKDIEALEYLSKVL---	108
<i>B. subtilis</i>	EHLLAFTIRSHAEKYDHF DI ISPMGCR TGF YLVVSSEPTSAEIVDLLED TM KEAV---	113
<i>H. pylori</i>	EHVLAETIRNHS----YVVDSPMGCR TGF YLVNHN DNY TEILEVLEK TM QDVL---	112
<i>C. jejuni</i>	DQSKIPELNIYQCGTCAMHSLDEAKQIAQKVLNLGISIINNKEKLKLEA-----	164
<i>V. harveyi</i>	NONKIPELNEYQCGTAAMHSLDEAKQIAKNILEVGVAVNKNDELALPESMLRELRI	172
<i>E. coli</i>	DQNKIPELNVYQCGTYQHSLQEAQDIARSLERDVIRNSNEELALPKEKLQELHI-	171
<i>H. influenzae</i>	DQASIPELNIYQCGSYTEHSLDAHEIAKNVIARGIVGNKNEDLSLNSLLK----	167
<i>P. multocida</i>	DQAKIPELNAPQCGTYTEHSLAEAQIAHNVLGRVSNKNEDLLDEQLLSL----	168
<i>C. perfringens</i>	EQEETPAANELQCGSAKLHSLAKSHAKVLENGISDKFYVE-----	151
<i>B. subtilis</i>	EITEIPAANEKQCGAKLHDLLEGAKRLMRFWLSQDKELKLVFG-----	157
<i>H. pylori</i>	KATEVPASNEKQCGGWAANHTLEGAKDLARAF LDK -RAEWS EVG V-----	155

Fig. 1. Alignment of the deduced *C. jejuni* *luxS* sequence with deduced *luxS* sequences from other bacterial species. *luxS* sequences from *C. jejuni* NCTC 11168 (SWISS-PROT Q9PN97), *V. harveyi* (SWISS-PROT Q9Z5X1), *E. coli* (SWISS-PROT P45578), *Haemophilus influenzae* (SWISS-PROT P44007), *Pasteurella multocida* (SWISS-PROT P57901), *Clostridium perfringens* (SWISS-PROT Q9XDU6), *Bacillus subtilis* (SWISS-PROT O34667) and *Helicobacter pylori* (SWISS-PROT Q9ZMW8) from BLASTP results were aligned using the CLUSTAL W algorithm (www.ebi.ac.uk). The amino acid residues that are conserved in all sequences of the alignment are marked in bold.

Fig. 2. The production of an extracellular signalling molecule with AI-2-like activity by *C. jejuni* NCTC 11168. *V. harveyi* BB170 was inoculated into AB medium containing 10% cell-free culture medium from *C. jejuni* NCTC 11168 (▲), 10% cell-free culture medium from *V. harveyi* BB152 (□) or 10% AB medium control (●). Cultures were incubated at 30 °C with constant agitation and aliquots were removed for measurement of luminescence. The results are a mean of four independent experiments. Bars indicate 1 SE of the mean.

(Fig. 2). Thereafter, at a cell density corresponding to the accumulation of a critical concentration of endogenously produced AI-2, bioluminescence increased dramatically (Fig. 2). In contrast, in the presence of cell-free culture preparations of *C. jejuni* NCTC 11168, bioluminescence did not decline but increased steadily over the course of the assay. This profile was similar to that generated by preparations taken from the AI-2 producing positive control, *V. harveyi* BB152 (Fig. 2). Since differences in the luminescence of *V. harveyi* cultures could not be attributed to variation in growth rates or differences in the cell density of the reporter strain (data not shown), these data suggest that *C. jejuni* produces an AI-2-like signalling activity.

Fig. 3. Production of the AI-2-like activity by *C. jejuni* NCTC 11168 is dependent on *luxS*. *V. harveyi* BB170 was inoculated into AB medium containing 10% cell-free culture medium from *V. harveyi* BB152 (□), 10% cell-free culture medium from *C. jejuni* NCTC 11168 (▲), 10% cell-free culture medium from *C. jejuni* NCTC 11168 (●), 10% cell-free culture medium from *E. coli* DH5α with pBAD-TOPO (△) and 10% cell-free culture medium from *E. coli* DH5α with pKE25 (●). The results are a mean of four independent experiments. Bars indicate 1 SE.

The *C. jejuni* signalling system is dependent upon LuxS

E. coli DH5α does not produce an AI-2 signal since it contains a frameshift mutation causing premature truncation of *luxS* (Surette *et al.*, 1999). To determine whether the *C. jejuni* LuxS orthologue Cj1198 could generate AI-2-like activity in a heterologous host, cell-free culture media prepared from *E. coli* DH5α containing pBAD-TOPO, and *E. coli* DH5α containing pKE25, which expresses Cj1198, were tested for the ability to induce luminescence in *V. harveyi* BB170 (Fig. 3). Whilst extracts from *E. coli* DH5α generated a similar response to that generated by AB medium alone, the addition of cell-free culture medium from cells containing pKE25 stimulated luminescence in a manner

similar to the positive control (*V. harveyi* BB152). This indicated that Cj1198 is able to generate an AI-2-like activity (Fig. 3). Consequently, we have now designated Cj1198 as LuxS. The orientation of the gene encoding Cj1198 in pKE25 is such that it can be transcribed from the inducible *araB* promoter. However, since AI-2-like activity was produced in the absence of arabinose induction of this promoter, this suggests that *luxS* is transcribed from its own promoter in this situation.

The gene encoding Cj1198 was disrupted by insertion of a kanamycin-resistance cassette (see Methods) and Southern blot analysis was used to confirm that a double homologous recombination event had occurred. Chromosomal DNA was isolated from the wild-type and three presumptive *luxS* mutants, digested with *XmnI* and then probed with a *luxS* containing DNA fragment. In DNA isolated from the wild-type strain, the probe hybridized to a single band of approximately 1800 bp (data not shown). In contrast, in all three presumptive mutant strains, the probe hybridized to a single band of approximately 3200 bp. This is consistent with the introduction of the disrupted *luxS* sequence, containing the 1.4 kb kanamycin-resistance marker, into the chromosome. Three independent mutants designated CJLUXS01–03 were chosen for further study. Whilst all three showed identical phenotypes, only the data obtained from CJLUXS01 are presented hereafter. Cell-free culture preparations taken from this mutant failed to stimulate bioluminescence in the assay for AI-2-like activity, in contrast to preparations made from wild-type *C. jejuni* cells (Fig. 3). Consequently, *luxS* is essential for the production of AI-2 activity in *C. jejuni*.

Production of the *C. jejuni* signalling molecule at different growth phases

The *V. harveyi* BB170 reporter was used to monitor production of the signalling molecule at different stages of growth of *C. jejuni* (Fig. 4). Maximal autoinducer activity was detected in cell-free conditioned medium after 18 h growth and remained at this level for at least 42 h. As expected, cell-free conditioned medium taken from CJLUXS01 did not stimulate luminescence in the reporter system for any growth phase (data not shown).

The phenotype of the *luxS* mutant

The microaerobic growth of CJLUXS01 in M-H broth was comparable to the parental strain in terms of doubling times (103 and 110 min, respectively) and time taken for entry into the stationary phase (20 h). Following exposure to air, a 3 log reduction in viable counts occurred in 12 and 10.5 h for *C. jejuni* NCTC 11168 and CJLUXS01, respectively, and no viable bacteria were detected after 24 h for either strain. The resistance of the two strains to oxidative stress was also equivalent as assessed using paraquat (1–40 mM) and hydrogen peroxide (0.3–0.015%, v/v) well diffusion assays (data not shown). Overall adherence and invasion of CJLUXS01 in Caco-2 cells was not significantly different from that of the parental strain (Mann–Whitney $P > 0.05$). The

Fig. 4. Production of *C. jejuni* signalling molecule at different growth phases. *C. jejuni* was cultured in M-H broth and aliquots were removed for measurement of viable counts (▲) and the capacity of cell-free conditioned medium to induce luminescence in *V. harveyi* BB170 at the 3 h time point (bars). Luminescence is shown as a ratio of relative light units in the presence of *C. jejuni* cell-free conditioned medium compared to relative light units in the presence of uninoculated M-H medium. The results are a mean of three independent experiments. Bars indicate 1 SE.

number of viable bacteria recovered after gentamicin treatment of infected Caco-2 cells was $5.6 \times 10^5 \text{ ml}^{-1}$ and $4.4 \times 10^5 \text{ ml}^{-1}$ for the parental and *luxS* mutant, respectively. Thus the proportion of the inoculum internalized by the Caco-2 cells was 0.08% for *C. jejuni* NCTC 11168 and 0.05% for CJLUXS01. When the *luxS* mutant and parental strain were tested for their ability to form motility haloes in semisolid agar, CJLUXS01 consistently gave rise to significantly ($P < 0.05$) smaller haloes using the Mann–Whitney test.

DISCUSSION

This study describes for the first time the production of a quorum-sensing molecule by *C. jejuni* NCTC 11168 and several lines of evidence suggest that this compound is closely related to the AI-2 family of signalling molecules. Cell-free culture fluids from *C. jejuni* contained a signalling molecule that activated the expression of bioluminescence in the *V. harveyi* BB170 reporter strain that responds to AI-2, but not AI-1 molecules (Bassler *et al.*, 1997). Sequence analysis of the entire genome sequence (Parkhill *et al.*, 2000) also revealed the presence of a LuxS orthologue (Cj1198). This protein was shown to exhibit similarity to LuxS from a number of different bacterial species. However, it is surprising that the *C. jejuni* protein showed greater identity with LuxS from *V. harveyi* (74%) and *E. coli* (71%) than with that from *H. pylori* (40%) given that *C. jejuni* and *H. pylori* are closer phylogenetic neighbours. Production of AI-2 in *C. jejuni* was found to be dependent on LuxS, since a null mutation in the corresponding gene rendered *C. jejuni* unable to induce bioluminescence in *V. harveyi* BB170 and the cloned *luxS* gene complemented the *luxS* frameshift mutation of *E. coli* DH5 α .

The pattern of AI-2 production with respect to growth phase and changes in environment may give some insight into the function of this signalling system. In this context, the growth-phase-dependent production of this molecule appears to vary with species. Accordingly, whilst the concentration of this signalling molecule is maximal in late-log or early-stationary-phase cultures in *Sal. typhimurium*, *E. coli* (Surette & Bassler, 1998; Surette *et al.*, 1999), *V. vulnificus* (McDougald *et al.*, 2001) and *Shi. flexneri* (Day & Maurelli, 2001), in *H. pylori* maximal production was shown in the early-log to mid-exponential phase and activity was diminished in stationary phase (Forsyth & Cover, 2000; Joyce *et al.*, 2000). AI-2 production is also responsive to perturbations in the metabolic activity of cells brought about by stress (DeLisa *et al.*, 2001). In *C. jejuni* NCTC 11168, maximal AI-2 production was induced in early-stationary-phase cultures and this activity remained at this elevated level for at least 42 h, suggesting that the *C. jejuni* signalling molecule is not degraded up to this point.

The systems used by bacteria to signal and sense their population density as well as the population density of other bacteria appear highly conserved. The responses to these environmental cues, however, are varied. Phenotypic responses not directly associated with pathogenesis include induction of bioluminescence in *V. harveyi* (Surette *et al.*, 1999), competence and sporulation in *B. subtilis* (Lazazzera & Grossman, 1998), biofilm formation by *P. aeruginosa* (Davies *et al.*, 1998; De Kievit *et al.*, 2001) and starvation survival in *V. vulnificus* (McDougald *et al.*, 2001). In pathogenesis, quorum sensing has been shown to regulate virulence factors (Parsek & Greenberg, 2000; De Kievit & Iglewski, 2000). Whilst the production of an AI-2 signalling molecule has been experimentally determined in a number of bacteria (Surette *et al.*, 1999; Joyce *et al.*, 2000; Day & Maurelli, 2001; McDougald *et al.*, 2001), and now *C. jejuni*, many features of the AI-2 family of quorum-sensing systems are not yet completely understood (Surette *et al.*, 1999). The structure of the AI-2 molecule has recently been predicted as a furanone and is produced by three enzymic steps from *S*-adenosyl-methionine, the final step being catalysed by the *luxS* gene product to give 4,5-dihydroxy-2,3-pentanedione which cyclizes into a furanone ring (Schauder *et al.*, 2001).

Whilst the function of the LuxS signalling systems remains unclear, some insights into its function have recently become apparent. In this context, LuxS has been shown to regulate the locus of enterocyte effacement (LEE) operon in enterohaemorrhagic *E. coli* O157:H7 and enteropathogenic *E. coli* (Sperandio *et al.*, 1999) and AI-2 activity has also been shown to be responsible for the late-exponential-phase peak of expression of VirB, a transcription factor essential for the expression of invasion loci in *Shigella flexneri* (Day & Maurelli, 2001). Inactivation of LuxS in the Gram-positive pathogen *Streptococcus pyogenes* and analysis of resulting mutants also suggested a role for LuxS in

modulation of virulence during infection (Lyon *et al.*, 2001). However, a *luxS*-deficient mutant of *H. pylori* had comparable growth, motility, urease and vacuolating cytotoxin activity in comparison to the wild-type (Forsyth & Cover, 2000; Joyce *et al.*, 2000). Quorum sensing in *P. aeruginosa* has been shown to contribute to the regulation of genes for relieving oxidative stress (Hassett *et al.*, 1999). *P. aeruginosa* mutants devoid of one or both HSL signalling molecules were more sensitive to hydrogen peroxide than the parental strain. The *luxS* mutant of *C. jejuni* NCTC 11168 showed comparable growth rate, resistance to oxidative stress and ability to invade Caco-2 cell monolayers to the parental strain. The invasiveness of *C. jejuni* NCTC 11168 in Caco-2 cell monolayers was comparable to published data for clinical *C. jejuni* isolates (Everest *et al.*, 1992; Harvey *et al.*, 1999). The CJLUXS01 mutant did, however, consistently give rise to smaller motility haloes than the parental strain, suggesting a role for quorum sensing in the regulation of motility. A similar role has been proposed for *E. coli* O157 since *luxS* mutants of this strain also showed smaller motility haloes (Sperandio *et al.*, 2001).

C. jejuni clearly uses a AI-2-based cell-signalling system but the function of this system is not yet fully known. This study has shown that it plays a role in motility; however, it is likely that this system also serves as a global regulatory mechanism for basic physiological functions and possibly virulence factors, as is the case in other bacterial pathogens.

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