Quorum sensing in Clostridium difficile: analysis of a luxS-type signalling system

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The increasing incidence of Clostridium difficile-associated disease, and the problems associated with its control, highlight the need for additional countermeasures. The attenuation of virulence through the blockade of bacterial cell-to-cell communication (quorum sensing) is one potential therapeutic target. Preliminary studies have shown that C. difficile produces at least one potential signalling molecule. Through the molecule’s ability to induce bioluminescence in a Vibrio harveyi luxS reporter strain, it has been shown to correspond to autoinducer 2 (AI-2). In keeping with this observation, a homologue of luxS has been identified in the genome of C. difficile. Adjacent to luxScd, a potential transcriptional regulator and sensor kinase, rolA and rolB, have been located. RT-PCR has been used to confirm the genetic organization of the luxScd locus. While AI-2 production has not been blocked so far using antisense technology, AI-2 levels could be modulated by controlling expression of the putative transcriptional regulator rolA. RolA, therefore, acts as a negative regulator of AI-2 production. Finally, it has been shown that the exogenous addition of AI-2 or 4-hydroxy-5-methyl-3(2H) furanone has no discernible effect on the production of toxins by C. difficile.

INTRODUCTION

Primarily a disease of the elderly, Clostridium difficile-associated disease is increasing concomitant with ageing Western populations (Spencer, 1998; Mylonakis et al., 2001). The cost implications to national health services worldwide are considerable (Miller et al., 2002; Mylonakis et al., 2001; Wilcox et al., 1996; Wilcox & Smyth, 1998; Riley, 1998), and highlight the need for additional countermeasures. One potential approach (Williams, 2002) would be to develop drugs that interfere with quorum sensing (QS). QS is the mechanism by which bacterial populations coordinate cell gene expression in response to cell population density (Fuqua et al., 1994). Small, diffusible signal molecules, which sometimes function as autoinducers (AI), produced by individual bacteria act as intercellular communication signals that are sensed by the population as a whole, resulting in the coordinate production of virulence factors. Such multicellular coordination is critical to the successful colonization/infection of the host by many pathogenic bacteria (Williams et al., 2000; Swift et al., 2001; Camara et al., 2002).

The AI paradigm in Gram-negative bacteria is N-acylhomoserine lactone (AHL). Molecules of this type are typically synthesized by enzymes belonging to the LuxI family first identified in Vibrio fischeri (Swift et al., 2001). LuxI utilizes the acyl chain of the appropriately charged acyl carrier protein (acyl-ACP) for AHL synthesis, while the homoserine lactone moiety is derived from S-adenosylmethionine. Signal specificity is conferred by the length, and the nature of the substitution at C-3, of the acyl side chain (Swift et al., 2001). In Gram-positive bacteria, the equivalent signals are often small post-translationally modified peptides. The best understood system is the agr (accessory gene regulator) locus of Staphylococcus aureus, which regulates the temporal production of cell-surface colonization factors and exoproteins, which contribute to virulence (Novick & Muir, 1999). The autoinducer peptide produced by S. aureus characteristically contains a thiolac-tone bond derived from the C-terminal carboxyl group bound to the thiol group of a highly conserved cysteine residue (McDowell et al., 2001).

More recently, a novel class of QS molecule, AI-2, has been identified. Its synthesis is reliant on the luxS gene, which itself forms part of the activated methyl cycle pathway (Winzer et al., 2003). This pathway is intimately linked to methionine metabolism, and is responsible for the generation of the methyl donor S-adenosylmethionine. Initially discovered in the marine bacterium Vibrio harveyi, AI-2 has subsequently...
been found to be produced by many different Gram-negative and Gram-positive species (Bassler et al., 1997) and has been implicated in the regulation of virulence in a number of human pathogens (Sperandio et al., 1999; Ohtani et al., 2002; Lyon et al., 2001; Burgess et al., 2002; Winzer et al., 2002b, 2003). Its ubiquity and apparent lack of specificity has led to the suggestion that it represents a general interspecies communication system (Bassler et al., 1997).

There is presently no direct evidence that C. difficile employs QS. However, it is clear that the production of its two major virulence determinants, toxin A and toxin B, is cell population density dependent, occurring late in the growth phase (Dupuy & Sonenshein, 1998). In Clostridium perfringens, toxin production has been shown to be under the control of an as-yet-unidentified AI, substance ‘A’ (Shimizu et al., 1997). More recently, AI-2 has been implicated in the production of virulence factors in C. perfringens (Ohtani et al., 2002). An inspection of the partial genome sequence of C. difficile 630 being undertaken at the Sanger Institute revealed the presence of a luxS homologue. The aim of this study was therefore to determine its role, if any, in the control of toxin production by C. difficile.

**METHODS**

**Bacterial strains and growth conditions.** Two strains of C. difficile were used in this study, CD630, the genome strain, and CD3, a clinical isolate, ribotype R7201. Both strains were routinely grown using BHI medium (Purdy et al., 2002) or TY medium at 37 °C in an anaerobic cabinet (MK3 Anaerobic Work Station; DW Scientific) containing an atmosphere of 80 % (v/v) nitrogen, 10 % (v/v) hydrogen and 10 % (v/v) carbon dioxide. C. difficile strains were maintained anaerobically at 37 °C as spores in cooked meat and carbohydrate.

Progression of growth of C. difficile cultures in liquid medium was monitored spectrophotometrically at OD600 using 1 ml samples in a Pharmacia Novaspec II.

**DNA manipulation techniques.** Plasmid isolation, agarose gel electrophoresis and transformation were performed as described by Sambrook et al. (1989). PCR amplifications were carried out using the Failsafe PCR kit (Cambio) according to the manufacturer’s instructions with buffer E (unless otherwise stated). Bacterial chromosomal DNA isolation and purification of DNA fragments from agarose gels was carried out using the Dneasy Tissue and Qiaquick Gel Extraction kits, respectively (Qiagen), according to the manufacturer’s instructions.

All DNA modification enzymes were used in the buffers and under conditions recommended by the manufacturer (New England Biolabs, unless otherwise stated).

**Vector construction.** Plasmid pGC1 was constructed as follows. The luxScd gene was amplified by PCR from C. difficile strain 630 chromosomal DNA using primers LSF3 (5′-GAGGTTTAATCTGCAATTTGCTAGCTG-3′) and LSR3 (5′-GAGCTAAATATGAAAGAAATG-3′) and contained Nhel and HindIII restriction sites, respectively. The 465 bp fragment was then subcloned into the equivalent sites of pBAD24, generating plasmid pGC1.

The GCeX expression cassette was constructed as follows. The lac promoter (Minton et al., 1990) was amplified by PCR as a 203 bp fragment using primers facF (5′-GTATATAGCATAGCTAAAGAGGAGATTC-3′) and facR (5′-GATTTCATAGCTAAAGAGGAGGGTC-3′) and cloned into pcR2.1 TOPO. The promoter was then excised as an EcoRI fragment and subcloned into the respective site of pMTL23 (Chambers et al., 1988), generating pMTL23F. Next, the Clostridium pasteurianum ferredoxin terminator was amplified as a 220 bp fragment excised from the C. pasteurianum genome using primers Fd1-2 (5′-GTAGTGATGAGATGAGAGGAGAGGAGGGTC-3′) and Fd-2 (5′-GTTTCCATGCTAGCTAAAGAGGAGGGTC-3′) and cloned into pcR2.1 TOPO. The fragment was then isolated as an XbaI fragment and subcloned into the XbaI site of pMTL23F located immediately upstream of the lac promoter, generating pMTL23FT. Finally, a 246 bp celA terminator fragment was amplified from Clostridium cellulolyticum using primers cel-1 (5′-CTGAGAGATTATGCTCAGTAAAGAGGAGGGTC-3′) and cel-2 (5′-AAATGCTGACCTTTTACAAACCAAGGAGGAGGGTC-3′) and cloned into the equivalent sites of pBAD24, generating plasmid pBAD24-Lux. All fragments containing MluI and BamHI restriction sites at the 5′- and 3′-ends, respectively, and were subcloned in the same way, using these sites and the equivalent sites of pMTL9341, generating plasmids pMTL9341aLS1, 2, 3 and 4 and pMTL9341aRR were constructed as follows. Gene fragments were amplified from CD630 DNA using primers aLS1F (5′-ACGCGTTGTTACTATGCTAGCAGTCAAGGA-3′) and aLS1R (5′-GGATCCTGATTTATGCTTATATCT-3′), producing a 532 bp product from 43 bp upstream of the start codon to 33 bp downstream of the stop codon; aLS2F (5′-ACGCGTTGTGATAAATATTTTATATTAAACAAAGG-3′) and aLS2R (5′-GGATCCTTTTCTACCCATTACAGTTAGTTTCAACATCGGAGGAGGAGGGTC-3′), producing a 346 bp fragment from 258 bp upstream of the start codon to nucleotide position 88 within the coding region of luxS; aLS3F (5′-ACCGTGGTTACTATGCTAGCAGTCAAGGA-3′) and aLS3R (5′-GGATCCTGCTCCTGCTAGTTTCAACATCGGAGGAGGAGGGTC-3′), producing a 569 bp fragment from 46 bp upstream of the start codon to nucleotide position 523 in the coding region of rolA. All fragments contained MluI and BamHI restriction sites at the 5′- and 3′-ends, respectively, and were subcloned in the same way, using these sites and the equivalent sites of pMTL9341, generating plasmids pMTL9341aLS1, 2, 3 and 4 and pMTL9341aRR.

**Al-2 assay.** The Al-2 bioluminescence assay was carried out essentially as described by Bassler et al. (1993). The V. harveyi reporter strain BB170 was grown overnight in LB medium before being diluted 1:5000 in AB medium containing 10 % (v/v) cell-free conditioned medium and allowed to grow at 30 °C with shaking. Controls consisted of AB medium containing 10 % (v/v) cell-free conditioned medium collected from Escherichia coli DH5α at negative, 10 % (v/v) from V. harveyi BB120 as positive, and 10 % (v/v) sterile BHI medium as a blank. Luminescence was measured every hour using a Wallac Victor2 1420 multilabel counter. Induction of luminescence was taken at the time point of the induction observed in the positive control.

**RNA isolation and RT-PCR.** C. difficile, 1 × 10^7 − 1 × 10^8 c.f.u. ml^-1, was lysed using the Gram Cracker Reagents kit (Ambion) according to the manufacturer’s instructions for lysis of Gram-positive rods. Total RNA was then extracted from the C. difficile lysate using Qiagen’s Rneasy Kit, as described in the manufacturer’s instructions. The
integrity and concentration of extracted RNA was determined spectrophotometrically using standard techniques (Sambrook et al., 1989) before use in RT-PCR. RT-PCR reactions were carried out with the OneStep RT-PCR kit (Qiagen) according to the manufacturer’s instructions using 50 ng total RNA. The negative control without reverse transcriptase was carried out in OneStep RT-PCR Buffer, but with the addition of HotStarTaq DNA Polymerase (Qiagen) rather than the OneStep RT-PCR Enzyme Mix. The following primers were used: rolAB overlap region, RR-HPK/For (5′-AGTTGGGATGATGTTAGTTA TAG-3′) and RR-HPK/Rev (5′-GCATTGAAATTTAAGCACAG-3′); rolB to luxScd intergenic region, HPFK3 (5′-GTTGAGGTTCTG GATTGG-3′) and HPKR3 (5′-CTTTATCTTCATCAAATACAC-3′); luxScd internal region, RT5 (5′-GTGACTGATGGAATAGGAGG-3′) and LS/Rev (5′-CTTCCTCTAAAACCAATCTTTA-3′); luxScd/orfX overlap region, LS/ORF-For (5′-GTGAGATTGTTAGAAGAAG-3′) and LS/ORF-Rev (5′-CTCTAATTCTCACAATTTACC-3′); orfX/ metHcd overlap region, MetH/ORF-For (5′-CAATTAGAAGAAGT GTGTGAC-3′) and MetH/ORF-Rev (5′-CATTGTGATCTAATACACATC-3′).

**Toxin A/B ELISA.** Toxin A/B levels were assayed by ELISA with a RIDASCREEN Clostridium difficile Toxin A/B kit (R-Biopharm) according to the manufacturer’s instructions. Prior to assay, the samples were diluted 1:100 in the universal stool buffer provided and 50 µl of this dilution was used. The plates were read at 450 nm in a Wallac Victor2 1420 multilabel counter.

**RESULTS**

**Identification of a luxS homologue in C. difficile strain 630**

Since the discovery of luxS as the gene responsible for production of AI-2 in V. harveyi, homologues of this gene have proven to be widespread amongst the bacterial kingdom. Accordingly, the V. harveyi LuxS protein was used in a BLASTP search against the C. difficile genome sequence (http://www.sanger.ac.uk/Projects/C_difficile/). This analysis identified a gene of 453 bp in length, capable of encoding a protein which shared 40 % identity and 57 % similarity to the V. harveyi protein and exhibited significant similarity to a number of other LuxS orthologues (Fig. 1). The protein consisted of 151 amino acids and was estimated to be approximately 15 kDa in size. The gene was designated luxScd.

Analysis of the region which preceded luxScd (Fig. 2) revealed the presence of a pair of adjacent ORFs capable of producing proteins with strong homology to a transcriptional regulator and a sensor kinase. Typical of a bacterial two-component signal transduction system, these genes were designated ‘regulator of luxS’ A and B (rolA and rolB). Downstream of luxScd an additional two ORFs are present. The first has been designated orfX, and its product shares no significant homology with any known protein. The second exhibits significant homology at the amino acid level with 5-methyltetrahydrofolate–homocysteine methyltransferase (MetH) of E. coli. It has therefore been designated metHcd. Interestingly, rolAB were found to overlap by 10 bp, as did luxScd and orfX by 13 bp and orfX and metHcd by 84 bp, suggesting that the five genes constitute an operon.

**AI-2 activity as a function of growth**

AI-2 activity was determined by the ability of cell-free supernatant from C. difficile strain 630 to induce bioluminescence in V. harveyi BB170. AI-2 activity was observed to increase gradually throughout the growth curve of C. difficile, with maximal induction achieved during the late–exponential stage of growth, after approximately 8 h (Fig. 3). At this time point cell-free supernatant was found to induce bioluminescence in the reporter to approximately 35 % of the level induced by wild-type V. harveyi BB120 supernatant. AI-2 activity subsequently diminished as the culture entered the stationary phase of growth. These observations are in agreement with those of others, who have shown AI-2 production to occur predominantly during the mid- to late–exponential phases of growth (Surette & Bassler, 1999; Day & Maurelli, 2001; Burgess et al., 2002).

**Complementation of E. coli DH5α with luxScd**

To determine whether luxScd encoded a functional AI-2 synthase, complementation studies were carried out in E. coli DH5α. This strain contains a frameshift mutation in luxScd and is therefore unable to produce AI-2 (Surette et al., 1999). To accomplish this, a DNA fragment encompassing the luxScd ORF was PCR-amplified from C. difficile 630 chromosomal DNA and subcloned into plasmid pBAD24, generating pGC1. Expression of luxScd from pGC1 could therefore be controlled by the addition of arabinose or glucose to the growth media, as described by Guzman et al. (1995). Plasmid pGC1 was electroporated into E. coli DH5α, and the recombinant strain was grown to mid-exponential phase in media supplemented with 0·05 or 0·2 % arabinose or 0·2 % glucose, after which point cell-free supernatants were tested for the ability to induce bioluminescence in V. harveyi BB170. The supernatant prepared from DH5α(pGC1) grown under inducing conditions (0·05 or 0·2 % arabinose) was able to activate bioluminescence in the reporter strain in a dose-dependent manner (data not shown). When grown in the presence of 0·05 % arabinose, supernatant was able to induce bioluminescence to levels of approximately 12 % of that of V. harveyi wild-type supernatant, whilst growth with 0·2 % arabinose resulted in levels of induction approximately 23 % of wild-type levels. In contrast, supernatant collected from wild-type E. coli DH5α and from E. coli DH5α(pGC1) grown under non-inducing conditions failed to induce bioluminescence in the V. harveyi reporter strain above background levels, indicating that luxScd does encode an active AI-2 synthase.

**Transcriptional analysis of the luxScd operon**

RT-PCR was used to map the luxScd operon region and to determine whether there is transcriptional linkage between any or all of the genes present within the locus. Fig. 2 indicates that rolA and rolB were present on a single RNA species, which was transcribed to higher levels during the early rather than the late stage of growth (as judged by the intensity of the products generated). The opposite was found to be true of
Fig. 1. Alignment of the predicted C. difficile LuxS sequence with the LuxS sequences from other bacterial species. The sequences were aligned using the CLUSTAL W algorithm. The amino acid residues that are conserved in all orthologues of the alignment are shown. The region between luxS and orfX, which was found to be transcribed at much higher levels during late-exponential growth, when AI-2 production was maximal.

The region encompassing orfX and metH, which was found to be transcribed on a single RNA species, which was apparently produced constitutively throughout early and late stages of growth (Fig. 2). Interestingly, using the very sensitive one-step RT-PCR kit, it was possible to amplify the intergenic region between rolB and luxS, as well as the overlapping region between luxS and orfX, which could indicate that these two loci are also transcriptionally linked. However, using a less sensitive RT-PCR kit, it was not possible to amplify either region.

Modulation of AI-2 activity using antisense RNA

It is currently not possible to generate insertionally inactivated mutants of C. difficile. We therefore opted to attenuate gene expression through the delivery of antisense RNA. To
achieve this, an expression cassette (GCex) based on the strong *C. pasteurianum* ferredoxin promoter was constructed. This was then subcloned into the conjugative, clostridial shuttle vector pMTL9301 (Purdy et al., 2002), generating pMTL9341. Several regions of the *luxS* Cd ORF and a 569 bp fragment from the 5’-region of *rolA* were amplified by PCR and subcloned into pMTL9341, in reverse orientation, generating pMTL9341aLS1-4 and pMTL9341aRR, respectively.

The plasmids were transferred to *C. difficile* strain CD3 (ribotype R7201) by conjugation as described previously by Purdy et al. (2002). Following transfer, the production of antisense RNA was confirmed by RT-PCR (data not shown). Cell-free supernatant was then collected throughout the growth curves of all recombinant strains, and the ability to induce bioluminescence in *V. harveyi* BB170 was assessed. In the strains producing antisense RNA targeted against the *luxS* Cd region no significant differences in the levels of AI-2 when compared to CD3 harbouring only pMTL9341 (and hence not producing antisense RNA) were observed (data not shown). However, supernatant from the strain generat-

**Fig. 2.** *C. difficile* luxS locus. (a) Presence upstream of two genes, *rolAB*, which encode a putative two-component signal transduction system. Downstream of *luxS* is *orfX*, which is of orphan status, and *methH* Cd, which is predicted to encode a 5-methyltetrahydrofolate-homocysteine methyltransferase. Bars below the genes illustrate the regions (1 – 5) amplified by RT-PCR. (b) RT-PCR was carried out on RNA extracted during early-exponential growth (e) and during late-exponential growth (l). Control reactions were carried out without reverse transcriptase (No RT) and with a DNA template (DNA).

**Fig. 3.** Growth and AI-2 production by *C. difficile* strain 630. *C. difficile* was grown anaerobically in BHI medium and aliquots were removed for OD600 readings (●). Cell-free supernatants from each time point were assayed for AI-2 activity using *V. harveyi* BB170 (bars). Bioluminescence is shown as a percentage of wild-type *V. harveyi* BB120 bioluminescence, which was assumed to be 100%.
ing antisense RNA targeted against rolA was consistently found to induce bioluminescence to levels approximately 12% higher than the pMTL9341 control at maximal levels (Fig. 4). Statistical analysis using the pairwise Student’s \( t \)-test showed this to be significant at a confidence interval of 95%. The data shown (Fig. 4) are the mean value from two independent experiments. In each case, percentage induction was calculated as the mean value of triplicate luminescence readings.

**Effect of AI-2 and 4-hydroxy-5-methyl-3(2H) furanone (MHF) on toxin production by C. difficile**

To determine whether AI-2 stimulated toxin production in *C. difficile*, cultures of CD630 were grown in TY medium supplemented with 25% *E. coli* DH5\(\alpha\)(pGC1) supernatant after growth in LB plus 0.2% arabinose to mid-exponential phase. This is an analogous experiment to that described for *C. perfringens* by Ohtani et al. (2002). As a negative control, a culture of CD630 was supplemented with 25% plasmid-free *E. coli* DH5\(\alpha\) supernatant after growth till mid-exponential phase. In addition, a further culture was supplemented with 25% LB plus 0.2% arabinose, in order to discount the presence of arabinose as the cause of any effect observed. Samples of each culture were collected after 24 and 48 h growth and the levels of toxin A and toxin B were determined by ELISA. Fig. 5 shows that growth in the presence of DH5\(\alpha\)(pGC1) supernatant containing AI-2 had no significant effect on the amount of toxin produced by CD630, when compared with the unsupplemented culture and the culture grown with DH5\(\alpha\) supernatant, which lacks AI-2.

To investigate further the effect of AI-2 on toxin production, CD630 was supplemented with 0, 1, 2 and 4 mM MHF, which is structurally related to AI-2 and induces bioluminescence in *V. harveyi* BB170, in the absence of AI-2 (Winzer et al., 2002c). In this series of experiments, samples were collected throughout the growth curve for up to 48 h and the amount of toxin A and toxin B was assayed. Fig. 6 shows the level of toxin A and B produced by CD630 after supplementation with MHF. These data are in agreement with the previous observations made using conditioned media, and show that the addition of MHF (AI-2) has no significant effect on the amount of toxin produced by *C. difficile*, when compared to CD630 grown in the absence
Our data suggest that in responsible for AI-2 production (increase in AI-2 production. To our knowledge, this is the production of antisense RNA resulted in a significant more importantly, modulation of luxS growth phase compared to early exponential. This reduction in mRNA levels mRNA being present when the cell population had achieved a early-exponential growth phase, with diminished levels of Cd mRNA were present in cells taken from late in the growth phase compared to early exponential.

The luxScd gene is immediately followed by an overlapping gene, orfX, of unknown function, which is in turn overlapped with a gene encoding a protein with homology to the MetH protein of E. coli. Superficially, this suggested that the three genes could form an operon. Indeed, using appropriate oligonucleotide primers, a weak PCR product encompassing the 3′-end of luxScd and the 5′-end of orfX could be generated using a very sensitive one-step RT-PCR kit. However, no such product was observed using a less sensitive kit. The fact that luxScd is transcribed primarily during later stages of growth and that the orfX region is constitutively transcribed is not consistent with the two genes being on the same RNA transcript. One possibility is that a bicistronic mRNA encompassing orfX and metH is produced constitutively, possibly from a promoter within the luxS coding region, whilst during exponential growth an additional tricistronic mRNA encoding luxScd, orfX and metH is produced from a second promoter upstream of luxScd. If this was the case, however, then the RNA species present during late-exponential growth should be twice the concentration of its early counterpart, and this does not appear to be the case.

A closer inspection of the region immediately preceding the translational initiation codon of orfX revealed the absence of any potential ribosome-binding sequences. This could indicate that orfX may not be expressed and may constitute a pseudogene. The RNA species encompassing luxScd and orfX may therefore represent an artefact of using the more sensitive one-step RT-PCR kit, or, if present, may be produced as a result of aberrant readthrough from the luxScd promoter. A similar explanation may be needed for the observed weak DNA product encompassing the intergenic

**DISCUSSION**

The experiments presented in this paper demonstrate that *C. difficile* 630 produces AI-2, or an AI-2 like molecule, capable of inducing bioluminescence in a *V. harveyi* AI-2 reporter strain. Through the complementation of an appropriate *E. coli* mutant, we have identified the gene most likely responsible for AI-2 production (luxScd). Maximal AI-2 activity is present during late-exponential phase, before it is rapidly depleted as the cells enter stationary phase. These data are in agreement with those of others (reviewed by Winzer *et al.*, 2003), including Surette *et al.* (1999) and Burgess *et al.* (2002), who showed similar phenomena in *Salmonella typhimurium* and *Porphyromonas gingivalis*, respectively. In keeping with the observed temporal regulation of AI-2 production, RT-PCR demonstrated that higher levels of luxScd mRNA were present in cells taken from late in the growth phase compared to early exponential.

The luxScd gene is preceded by a pair of genes (rolA and rolB) that encode proteins possessing features typical of bacterial two-component signal transduction systems. Such regulatory elements are known to play a key role in the regulation of peptide QS signal molecules in various Gram-positive bacteria (Kleerebezem *et al.*, 1997; Sturme *et al.*, 2002). Our data suggest that in *C. difficile*, the co-transcribed genes rolA and rolB are indeed involved in the regulation of AI-2 production in *C. difficile*. There are two lines of evidence for this conclusion. Firstly, in direct contrast to data obtained with luxScd, rolAB were more highly transcribed during early-exponential growth phase, with diminished levels of mRNA being present when the cell population had achieved a high population density. This reduction in mRNA levels coincided with a time point when AI-2 production and luxScd mRNA levels were both at their highest. Secondly, and more importantly, modulation of rolA expression through the production of antisense RNA resulted in a significant increase in AI-2 production. To our knowledge, this is the first report detailing the successful use of antisense RNA in *C. difficile*. Taken together, these data suggest that RolA is a negative regulator of LuxS and hence AI-2 production in *C. difficile*. Whether this regulation is through the direct interaction of RolA with luxScd or via intermediary proteins in a global regulatory cascade remains to be elucidated.
region between rolB and luxS_{CD}, particularly as its presence is not consistent with the differential transcription of rolAB and luxS_{CD}. The product of metH is known to be involved in the activated methyl cycle, which is an important pathway in methionine recycling (Winzer et al., 2003). In a number of organisms, luxS homologues have been found in close proximity to other genes associated with this metabolic pathway. For example, in C. perfringens, luxS_{CD} was found to be transcribed as part of a polycistronic mRNA along with two other genes, encoding metB and cysK (Banu et al., 2000), whilst in P. gingivalis, luxS_{PG} is suggested to be part of an operon along with a homologue of the E. coli 5-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase (Pfs) protein (Burgess et al., 2002). These findings in conjunction with other compelling evidence have led to the suggestion that in organisms other than V. harveyi, LuxS is simply a metabolic protein that fulfils a function analogous to that of SAH hydrolase in eukaryotes, archaea and some eubacteria, and that Al-2 far from being a QS autoinducer is merely a metabolic by-product generated from the conversion of S-ribosylhomocysteine (RH) to homocysteine (Winzer et al., 2002b, 2003). Furthermore, it is proposed that Al-2 is transported from the cell for reasons associated with its predicted toxicity rather than for cell-to-cell communication (Winzer et al., 2002a, 2003).

A more conclusive assignment of function is ultimately reliant on being able to insertionally inactivate the gene in question. There is so far only one such report in C. difficile (Liyanage et al., 2001), where inactivation of gldA in the non-pathogenic strain CD37 proved lethal. However, we have so far been unable to reproduce this method with either rolA or luxS_{CD}. Despite the successful use of antisense technology to modulate rolA expression, and the successful use of such strategies in other clostridial species (Desai & Papoutsakis, 1999; Tummala et al., 2002; Perret et al., 2004), we were unable to elicit any discernible effect on luxS_{CD}.

In the absence of a mutant it is not possible to determine whether the C. difficile luxS_{CD} is involved in QS, metabolism or another as yet unknown function. However, the fact that neither exogenous Al-2 nor MHF had any discernible effect on toxin production suggests that C. difficile does not use this ‘autoinducer’ to control toxin production. This is not entirely surprising, since the timing of toxin production in C. difficile is primarily after the organism has entered stationary-phase growth. As such, if LuxS/Al-2 do represent a true QS cascade in C. difficile, then it is not immediately obvious how they would be utilized to coordinate the production of toxins, which are produced almost exclusively after the autoinducer has become greatly depleted. It is possible that Al-2 could act as a negative switch, with the sudden loss of the molecule, rather than its reaching a critical threshold concentration being responsible for the induction of toxin production. However, whether such a system would meet the criteria needed to be termed ‘quorum sensing’ (as recently refined by Winzer et al., 2002a) seems unlikely. Although LuxS has been implicated in the pathogenesis and control of virulence factor production in a number of bacterial species, including alpha-, kappa- and theta-toxin production by C. perfringens (Ohtani et al., 2002), Rgp and KgP protease production by P. gingivalis (Burgess et al., 2002) and the regulation of the locus of Enterocytozoon Effacacement operons of E. coli O157:H7 (Sperandio et al., 1999), mutation of luxS often appears to have little effect on ‘real’ virulence, as is the case for P. gingivalis (Burgess et al., 2002), Shigella flexneri (Day & Maurelli, 2001) and Proteus mirabilis (Schneider et al., 2002). This has led to speculation that in the majority of organisms other than V. harveyi, analysis of luxS mutants has resulted in the identification of phenotypes not directly linked to QS, but rather to a reduction in fitness associated with the disruption of the activated methyl cycle at the level of LuxS (Winzer et al., 2002a, 2003).

Despite this, until an isogenic mutant can be generated in C. difficile and thoroughly tested, the role of LuxS and AI-2 in the pathogenesis or metabolism of C. difficile can not be conclusively determined.

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The Clostridium difficile luxS gene


