Regulation of autoinducer 2 production and *luxS* expression in a pathogenic *Edwardsiella tarda* strain

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Edwardsiella tarda is a bacterial pathogen that can infect both humans and animals. TX1, an *Ed. tarda* strain isolated from diseased fish, was found to produce autoinducer 2 (AI-2)-like activity that was growth phase dependent and modulated by growth conditions. The gene coding for the AI-2 synthase was cloned from TX1 and designated *luxS_{Et}*. *LuxS_{Et}* was able to complement the AI-2 mutant phenotype of *Escherichia coli* strain DH5 α . Expression of *luxS_{Et}* correlated with AI-2 activity and was increased by glucose and decreased by elevated temperature. The effect of glucose was shown to be mediated through the cAMP-CRP complex, which repressed *luxS_{Et}* expression. Overexpression of *luxS_{Et}* enhanced AI-2 activity in TX1, whereas disruption of *luxS_{Et}* expression by antisense RNA interference (i) reduced the level of AI-2 activity, (ii) impaired bacterial growth under various conditions, (iii) weakened the expression of genes associated with the type III secretion system and biofilm formation, and (iv) attenuated bacterial virulence. Addition of exogenous AI-2 was able to complement the deficiencies in the expression of TTSS genes and biofilm production but failed to rescue the growth defects. Our results (i) demonstrated that the AI-2 activity in TX1 is controlled at least in part at the level of *luxS_{Et}* expression, which in turn is regulated by growth conditions, and that the temporal expression of *luxS_{Et}* is essential for optimal bacterial infection and survival; and (ii) suggested the existence in *Ed. tarda* of a *LuxS*/AI-2-mediated signal transduction pathway that regulates the production of virulence-associated elements.

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INTRODUCTION

Quorum sensing (QS) is a complex process by which cell-cell communication is achieved in bacteria (Gonzalez & Keshavan, 2006). For Gram-negative bacteria, QS systems can be classified into several categories based on the type of autoinducers and relaying molecules involved. One of these, as exemplified by that observed with *Vibrio harveyi* in the regulation of bioluminescence production (Bassler *et al.* 1993; Mok *et al.*, 2003), employs autoinducer-2 (AI-2) as the signalling molecule. The *V. harveyi* AI-2 is produced from *S*-adenosylhomocysteine (SAH) via enzymic steps involving the nucleosidase Pfs, which converts SAH to *S*-ribosylhomocysteine (SRH), and *LuxS*, which converts SRH to 4,5-dihydroxy-2,3-pentanedione, the immediate precursor of AI-2 (De Keersmaecker *et al.*, 2006; Lewis *et al.*, 2001; Schauder *et al.*, 2001). Unlike

AI-1, which is generally species-specific and is responded to only by cells of kindred genetic lineages, AI-2 has been discovered to exist in diverse bacteria and the AI-2 molecules produced by one bacterial species are responded to by bacteria of different species and genera. Consistent with its proposed role as an interspecies communication signal, AI-2-mediated signal transduction circuits have been found to regulate biological processes that coordinate the behaviour of cells as a community to best adapt to various environmental situations. Such biological processes include biofilm formation, conjugation, sporulation, and production of virulence elements (Gonzalez Barrios *et al.*, 2006; McNab *et al.*, 2003; Rickard *et al.*, 2006; Surette *et al.*, 1999); the last has been linked to the *LuxS*/AI-2 quorum-sensing system via, most often, mutational studies of the *luxS* gene (Lyon *et al.*, 2001; Marouni & Sela, 2003; Ohtani *et al.*, 2002; Stroehrer *et al.*, 2003; Winzer *et al.*, 2002b; Zhu *et al.*, 2002).

Edwardsiella tarda is a Gram-negative bacterium that can be pathogenic to a broad range of host, including humans. Currently the major concern raised by this bacterium is its ability to cause edwardsiellosis, a systemic disease that has

Abbreviations: AI-2, autoinducer 2; CRP, cAMP receptor protein; EMSA, electrophoretic mobility shift assay; i.p., intraperitoneally; QS, quorum sensing; RITT, rho-independent transcriptional terminator; TTSS, type III secretion system.

The GenBank/EMBL/DDBJ accession number for the sequence of the *luxS_{Et}* region is EU070919.

been reported to occur in different parts of the world. Although recognized as one of the leading pathogens that pose a serious threat to the development of aquaculture industries worldwide, *Ed. tarda* has not been studied in a scope merited by its importance, and many fundamental processes, especially those concerning bacterial infection and survival, remain to be elucidated. Recently Morohoshi *et al.* (2004) identified the LuxI/LuxR homologues EdwI/EdwR in *Ed. tarda*, and suggested that, as in many other pathogens, the EdwI/EdwR system probably regulates the production of certain virulence factors.

We present in this report the study of AI-2 activity and *luxS* expression in *Ed. tarda* strain TX1, a fish pathogen. Our results indicate that the AI-2 activity in TX1 is controlled at the level of *luxS* expression, which is modulated by growth conditions. By using antisense RNA interference we obtained evidence supporting the idea that there exists in TX1 an active LuxS/AI-2-mediated QS pathway that is involved in bacterial pathogenicity.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Unless otherwise indicated, all strains were grown in Luria–Bertani (LB) medium at 37 °C (for *Escherichia coli*) or 28 °C (for all others) (Zhang & Sun, 2007). Appropriate antibiotics and supplements were added at the following

concentrations: ampicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; tetracycline, 15 µg ml⁻¹; X-Gal, 40 µg ml⁻¹. The mean generation time (g) of bacterial growth was calculated as described by Eagon (1962).

DNA and molecular techniques. Plasmid preparation, PCR amplifications, purification of PCR products and genomic DNA preparation were carried out as described previously (Zhang & Sun, 2007). Restriction endonucleases and modifying enzymes were purchased from New England BioLabs (China) and used in accordance with the manufacturer's specifications.

Plasmid constructions. The plasmids used in this study are listed in Table 1; primers are listed in Table 2. To construct pBT, the *rrnB* transcription terminator of pTrcHis (Invitrogen) was ligated into pBR322 between the *EcoRV* and *BsaBI* sites, resulting in plasmid pBRB. The *P_{trc}* promoter of pTrcHis was amplified by PCR with primers TrcF2/TrcR1 and inserted into pBRB between the *EcoRI* and *BamHI* sites, yielding plasmid pBT. pBTES was generated by inserting *luxS_{Et}* (amplified by PCR with primers F19/R17) into the *SmaI* site of pBT. To create pSC11, an *EcoRI*–*EcoRV*–*BamHI* linker was inserted into pSC6 between the *EcoRI* and *BamHI* sites, resulting in pSC7, which was then cut with *EcoRV*/*PvuII* and ligated to a promoterless *lacZ* gene amplified from TOP10ΔRS65 with primers LacF2/LacR3. To construct pSC100, the *P_{luxS}*-containing 100 bp DNA (named D100) immediately upstream of the translational start of *luxS_{Et}* was amplified by PCR with primers F11/R9 and the PCR products were ligated into pSC11 at the *SwaI* site. Similarly, pSC100M was created by inserting D100 containing the mutated *P_{luxS}* (amplified by PCR with primers F17/R9) into pSC11 at the *SwaI* site. pJRSN was created by first inserting the *bla* gene of pBR322 into pDN18 at the *EcoRI* site, resulting in plasmid pJRA, which was then digested with *EcoRV* and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
<i>E. coli</i>		
BL21(DE3)	Host strain for <i>crp</i> expression	Zhang & Sun (2007)
DH5α	Host strain for general cloning	Zhang & Sun (2007)
TOP10ΔRS65	Source of the <i>lac</i> operon	Sun <i>et al.</i> (1998)
<i>Ed. tarda</i>		
TX1	Tc ^R , fish pathogen	This study
<i>V. harveyi</i>		
BB120	Parental strain of BB170	ATCC
BB170	<i>luxN</i> ::Tn5; AI-1 defective	ATCC
Plasmids		
pBT	Ap ^R , pBR322 derivative carrying <i>P_{trc}</i>	This study
pBTES	Ap ^R , pBT expressing <i>luxS_{Et}</i>	This study
pET258	Kn ^R , expression plasmid	Zhang & Sun (2007)
pECRP	Kn ^R , pET258 expressing <i>E. coli crp</i>	This study
pDN18	Broad-host-range vector	Marx & Lidstrom (2001)
pJRA	Ap ^R , pDN18 derivative	This study
pJRSN	Ap ^R , pJRA expressing <i>luxS_{Et}</i>	This study
pJR18	Ap ^R , pJRA expressing <i>luxS_{Et}</i> antisense RNA	This study
pJZL	Ap ^R , pJRA carrying <i>P_{luxS}</i> – <i>luc</i> fusion	This study
pJZS	Ap ^R , pJRA carrying <i>P_{luxS}</i> – <i>lacZ</i> fusion	This study
pSC6	Kn ^R , cloning vector	Sun <i>et al.</i> (1998)
pSC11	Kn ^R , pSC6 derivative	This study
pSC100	Kn ^R , pSC11 carrying <i>P_{luxS}</i>	This study
pSC100M	Kn ^R , pSC11 carrying mutant <i>P_{luxS}</i>	This study

*Ap, ampicillin; Kn, kanamycin; Tc, tetracycline.

Table 2. Primers used in this study

Primer	Sequence (5'–3')*
CRPF1	GTGTCATATGGTGTGGCAAACCG (<i>Nde</i> I)
CRPR1	ATATCTCGAGACGAGTGCCGTAAACGAC (<i>Xho</i> I)
F1	GCGCTGCAGTTCGGGT
F10	GATATCCGCTGACGCTGTTGGA (<i>Eco</i> RV)
F11	ATGATAACAAATGCGCGTCT
F17	ATGATAACAAATGCGCGTCTTTCAGATACTGAGAGTCGCAG
F19	GATATCGCAGTATGGGAAAAGGT (<i>Eco</i> RV)
LacF2	TATCCCGGGATTAAATTTACACAGGAAACAGC (<i>Sma</i> I- <i>Swa</i> I)
LacR3	TTATCCCGGGTTATTTTGACACCAGACCA (<i>Sma</i> I)
R1	AAACCGGTGCGGCAGCCCAT
R9	GATATCGTCACCTCCTCAATAA (<i>Eco</i> RV)
R17	GATATCGGTGAAGCCTAGGG (<i>Eco</i> RV)
R18	GATATCCGGCAGGATGGCAT (<i>Eco</i> RV)
TrcF2	ATAGAATTCATTAAATCACTGCATAATTCGTG (<i>Eco</i> RI- <i>Swa</i> I)
TrcR1	TATTGGATCCATTATACGAGCCGGATG (<i>Bam</i> HI)

*Underlined nucleotides are restriction sites of the enzymes indicated in parentheses.

ligated to the *Swa*I fragment carrying the P_{trc} - $luxS_{Et}$ fusion of pBTES. pJR18 was constructed by inserting the antisense RNA of $luxS_{Et}$ (amplified by PCR with primers R18/F19) into the *Sma*I site of pBT, resulting in pBTSR; the *Swa*I fragment of pBTSR containing the P_{trc} - $luxS$ antisense RNA coding region was inserted into the *Eco*RV site of pJRA, yielding pJR18. pJZS was created by first inserting the promoterless *lacZ* gene into pACYC177 (New England BioLabs) at the *Sma*I site, resulting in plasmid p178, which was then digested with *Sma*I and ligated to P_{luxS} (generated by PCR with primers F10/R9), resulting in plasmid p178LS; the *Sma*I fragment of p178LS containing the P_{luxS} -*lacZ* fusion was inserted into pJRA at the *Eco*RV site. To create pJZL, the luciferase gene (*luc*) of pGL3-Basic (Promega) was inserted into pET28 (Novagen), resulting in pGL28; *luc* with the ribosome-binding site was amplified by PCR from pGL28 and inserted into pSC7 between the *Eco*RV and *Pvu*II sites, yielding pSC17, which was then digested with *Swa*I and ligated to P_{luxS} , resulting in pJZL.

Cloning of the $luxS_{Et}$ gene. This was carried out in a two-step process: first the DNA region internal to $luxS_{Et}$ was cloned by degenerate PCR using the primer pair F1/R1 derived from the sequences conserved among the known *luxS* genes; secondly the up- and downstream regions of the cloned $luxS_{Et}$ fragment were obtained by genome walking as described previously (Zhang & Sun, 2007).

Real-time reverse transcriptase (RT) PCR. Total RNA was extracted from fish organs and from bacterial cells grown in appropriate medium to OD₆₀₀ 1 by using the SV total RNA isolation system (Promega). Real-time RT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems) by using the SYBR ExScript RT-PCR kit (Takara, China). Each assay was performed in triplicate with 16S rRNA as a control. Dissociation analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. The comparative CT ($2^{-\Delta\Delta CT}$) method was used to analyse the mRNA level. All data are given in terms of relative mRNA expressed as means $\pm$ SEM. Statistical analyses were performed by using the two-tailed *t*-test.

Preparation of recombinant CRP. The *E. coli crp* gene was amplified by PCR with primers CRPF1/CRPR1 and the PCR products were ligated into pET258 between the *Nde*I and *Xho*I sites, resulting in

pECRP, which was introduced into the *E. coli* BL21(DE3) by transformation. The recombinant CRP was purified from BL21(DE3)/pECRP by using nickel-NTA beads as described previously (Zhang & Sun, 2007).

Gel electrophoresis mobility shift assay (EMSA). The 288 bp DNA fragment containing P_{luxS} was generated by PCR with primers F10/R9 and labelled with carboxyfluorescein. The labelled DNA was mixed with the purified recombinant CRP and incubated at 37 °C for 20 min in CRP binding buffer (Sun *et al.*, 2004) with or without 400 nM cAMP. After the reaction, the samples were run on a non-denaturing 8% polyacrylamide gel. As a negative control the 200 bp DNA region upstream of the *Eco*RI site of pBR322 was included in the assay.

AI-2 assay. The AI-2 assay was performed essentially as described by Surette & Bassler (1999). To prepare cell-free culture fluids, overnight cultures of cells grown in LB medium at 28 °C were diluted 1:100 in fresh LB medium; 2 ml of cell culture was taken every 30 min and the cell-free supernatant was obtained by centrifugation followed by filtering through a 0.22 μ m filter (Millipore). For measurement of bioluminescence induction, an overnight culture of *V. harveyi* strain BB170 grown in AB medium at 28 °C was diluted 1:5000 in fresh AB medium supplemented with cell-free culture fluids (10%) of the tested strains or with growth medium (as the control). Growth was continued and light production was measured by using a Glomax luminometer (Promega).

Complementation by exogenous AI-2. *Ed. tarda* TX1 was cultured in LB medium to OD₆₀₀ 1 and cell-free culture fluids (prepared as above) were added at a concentration of 10% to the growth medium of the cells under examination.

Biofilm development assay. Biofilm formation on a polystyrene surface was determined exactly as described by Xu *et al.* (2006).

Bacterial conjugation. pJRA and its variants were introduced into *E. coli* S17-1 λ pir (Biomedal) by transformation. The transformants and *Ed. tarda* TX1 were grown in LB medium to OD₆₀₀ 1 and mixed in a 1:1 ratio. The mixed cells were washed and resuspended in 10 mM MgSO₄ and dropped onto an LB plate. After incubation at

28 °C for 12 h, the growth on the plate was scraped off and resuspended in 1 ml LB, from which 100 µl was taken and spread onto a LB plate supplemented with ampicillin and tetracycline. The plate was incubated at 28 °C for 48 h and the colonies that appeared were verified to be authentic transconjugants by PCR and sequence analysis of the PCR products.

β -Galactosidase assay. This was performed as described by Sun *et al.* (1998).

Animal model study. Japanese flounders (*Paralichthys olivaceus*) (~14 g) were divided randomly into several groups (40 fish/group). Each group was injected intraperitoneally (i.p.) with the test bacterium that had been cultured to OD₆₀₀ 0.5 in LB medium, washed, and resuspended in phosphate-buffered saline (PBS). The animals were monitored for mortality in the 10 days post-infection and the accumulated mortalities were calculated. To examine bacterial dissemination, the blood and organs of the infected fish were removed under sterile conditions. The organs were homogenized with glass homogenizers and plated on LB plates containing selective antibiotics. The blood was plated directly. The plates were incubated at 28 °C for 48 h and the colonies that emerged were examined for TX1 harbouring or not harbouring plasmid by PCR using TX1-, pJRA- and pJ18-specific primers and subsequent sequencing of the PCR products. Statistical analyses were performed by the two-tailed *t*-test.

Database searching and nucleotide sequence accession number. Database searching was conducted using the BLAST programs at the NCBI (National Center for Biotechnology Information). The nucleotide sequence of the *luxS_{Et}* region has been deposited in the GenBank database under accession number EU070919.

RESULTS

Cell-free culture fluids of TX1 exhibit AI-2-like activity which is growth phase dependent and modulated by growth conditions

To investigate whether *Ed. tarda* strain TX1 produced active AI-2, the cell-free culture fluids of this strain, which was isolated from diseased fish, was analysed for the ability to stimulate light production in *V. harveyi* strain BB170, a reporter of AI-2 signalling (Bassler *et al.*, 1993). As shown in Fig. 1(a), TX1 possessed AI-2 activity that, up to the early exponential phase, increased with the progress of the growth in a manner similar to that observed with BB120, the wild-type strain from which BB170 is derived. After the cell density exceeded OD₆₀₀ 0.75, however, the AI-2 activity in BB120 declined sharply whereas that in TX1 continued to increase until the cell density reached OD₆₀₀ 1, where the AI-2 activity peaked. The maximum fold induction of light production effected by the culture fluids of TX1 was 35 % lower than that effected by BB120, suggesting that either the amount of active AI-2 present in TX1 is less than that in BB120 or the AI-2 of TX1 is less effective in the activation of the specific QS pathway in BB170. To determine whether the AI-2 activity of TX1 was affected by growth conditions, TX1 was cultured at 28 °C in LB with or without 0.5 % glucose to OD₆₀₀ ~0.95 or in LB medium at 37 °C to OD₆₀₀ ~0.65, which was close to the maximum cell density under the specific growth condition.

Fig. 1. (a) AI-2 activity in *Ed. tarda* TX1 and in *E. coli* DH5 α carrying pBT variants. Cell-free culture fluids of TX1, DH5 α harbouring pBT or pBTES, and *V. harveyi* BB120 were taken at various growth points and assayed for AI-2 activity. DH5 α /pBT showed no AI-2 activity (not plotted). (b) AI-2 activity in TX1 under different growth conditions. TX1 was grown in LB medium at 28 °C (left) or in LB medium at 37 °C (right) or at 28 °C in LB supplemented with 0.5 % glucose (centre). The cells used for AI-2 assay were harvested at OD₆₀₀ ~ 0.95 except for those grown at 37 °C, which were harvested at OD₆₀₀ 0.65. In both (a) and (b), AI-2 activities are presented as fold induction over the control. Data are representative of at least three independent experiments and presented as the means $\pm$ SEM. **, *P* < 0.01.

Subsequent AI-2 assay indicated that the AI-2 activity was significantly augmented by glucose and reduced by high temperature (Fig. 1b).

TX1 possesses a *luxS* homologue that can complement the AI-2-defective phenotype of *E. coli* DH5 α

The presence of AI-2-like activity in TX1 implied the existence in this strain of a functional *luxS* homologue. This *luxS* gene, named *luxS_{Et}*, was obtained from TX1 by degenerate PCR. The deduced amino acid sequence encoded by *luxS_{Et}* shares the highest identity (81 %) with the LuxS of *Erwinia carotovora* and possesses an HXXEH motif, which is a conserved LuxS signature. To examine whether *luxS_{Et}* encoded a functional enzyme, *E. coli* strain DH5 α , which is deficient in AI-2 synthesis (Surette & Bassler, 1998), was transformed separately with plasmid pBTES, which constitutively expresses *luxS_{Et}*, and the control plasmid pBT. The culture fluids of the transformants were then subjected to AI-2 assay, which showed that the presence of pBTES enabled the host cells to produce bioluminescence whereas that of pBT failed to do so (Fig. 1a).

Identification of the *luxS_{Et}* promoter

On the chromosome, *luxS_{Et}* is preceded by an ORF designated *gcl*; as there is a putative rho-independent transcriptional terminator (RITT) between *gcl* and *luxS_{Et}* (Fig. 2), we speculated that the promoter of *luxS_{Et}* was

probably located downstream of the RITT. Sequence inspection identified a putative σ^{70} -dependent promoter (named P_{luxS}) located 31 bp downstream of the RITT (Fig. 2). P_{luxS} was cloned into the promoter-probe plasmid pSC11, a low-copy-number plasmid with a pSC101 replication origin and a promoterless *lacZ* gene as the reporter of heterologous promoter activity. The recombinant plasmid, pSC100, was introduced into DH5 α by transformation. The transformant gave blue colonies on X-Gal plates and produced detectable β -galactosidase activity (44 Miller units), suggesting that P_{luxS} was an active promoter. In support of this, DH5 α transformed with pSC100M, which is identical to pSC100 except that the first T in the -10 region of P_{luxS} is 'down'-mutated to G, produced no detectable β -galactosidase activity.

Expression of $luxS_{Et}$ is growth phase dependent and regulated by growth conditions

We next investigated whether growth phase and growth conditions had any effect on $luxS_{Et}$ expression by real-time RT-PCR using RNA extracted from TX1 grown to different cell densities and under different conditions. The results showed that the expression of $luxS_{Et}$ increased as growth progressed and peaked at a cell density of OD₆₀₀ 1 (Fig. 3a). Glucose and high temperature caused, respectively, a 7.9-fold increase and a 7.6-fold decrease in $luxS_{Et}$ expression (Fig. 3b); hence there appeared to be a correlation between $luxS_{Et}$ expression and AI-2 activity (compare Fig. 1b). To further investigate whether this was true, we examined the effect of $luxS_{Et}$ overexpression upon AI-2 activity. To this end, the plasmid pJRSN, which constitutively expresses $luxS_{Et}$ and the control vector pJRA were each introduced into TX1 via conjugation. Overexpression of $luxS_{Et}$ in the transconjugant, TX1/pJRSN, was confirmed by real-time RT-PCR (data not shown). AI-2 assay indicated that the maximum fold stimulation effected by the culture fluids of TX1/pJRSN was 60 % higher than that effected by TX1/pJRA, suggesting that overexpression of $luxS_{Et}$ enhanced AI-2 production. These results, together with those of the above sections, supported the idea that in TX1 the AI-2 activity was controlled at the level of $luxS_{Et}$ expression and that the effects of glucose and temperature upon AI-2 activity were manifestations of the effects of these factors on $luxS_{Et}$ expression.

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TAGaaaagaaaaaggccacaatacctgtggccaaaataaa
catctctgaggacagggatgatGATAAcaaatgcgcg
-35
tcttcaTATACTgagatgocagatgggaaaagggttctc
-10
aacaagggaataatattttattgaggaggtgacgaaATG

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Fig. 2. Nucleotide sequence of the intergenic region between *gcl* and $luxS_{Et}$. The translation stop and start codons of *gcl* and $luxS_{Et}$ respectively, are in bold capital letters; the putative RITT is italicized; the putative CRP-binding site is underlined; the -10 and -35 elements of P_{luxS} are capitalized and indicated.

Fig. 3. Expression of $luxS_{Et}$ in relation to growth phase (a) and growth conditions (b). (a) Total RNA was extracted from *Ed. tarda* TX1 grown to different growth phases and used for real-time RT-PCR. (b) Total RNA was extracted from strain TX1 grown to OD₆₀₀ 0.9 in LB medium at 28 °C or in LB with the indicated modifications (i.e. at 37 °C, in the presence of 0.5 % glucose, 5 mM cAMP, or glucose plus cAMP) and used for real-time RT-PCR. The $luxS_{Et}$ mRNA level was normalized to that of the 16S rRNA. Data are presented as the means $\pm$ SEM. **, $P < 0.01$; *, $P < 0.05$.

In vivo transcription from P_{luxS} is regulated by growth conditions

To further examine the effect of growth conditions upon transcription from P_{luxS} in TX1, the conjugative plasmid pJZS, in which P_{luxS} directs the expression of a *lacZ* reporter gene, was conjugated into TX1. The transconjugant, TX1/pJZS, was assayed for β -galactosidase production; its β -galactosidase activity, which was 5.8 Miller units when grown in LB medium at 28 °C, was increased fivefold (33 Miller units) by glucose and rendered undetectable by high temperature (37 °C). These results indicated that in TX1 P_{luxS} activity correlated with $luxS_{Et}$ expression and, therefore, P_{luxS} was very likely the actual promoter of $luxS_{Et}$.

The effect of glucose upon $luxS_{Et}$ expression is mediated through the cAMP-CRP complex

Since glucose can decrease the cellular cAMP level and thus affect the activity of the cAMP receptor protein (CRP), we determined whether cAMP had any effect upon $luxS_{Et}$ expression by real-time RT-PCR. The results showed that the presence of cAMP (5 mM) alone significantly repressed $luxS_{Et}$ expression whereas cAMP combined with glucose completely abolished the stimulating effect of glucose (Fig. 3b). A similar cAMP effect was also observed with AI-2 activity (data not shown). In line with these observations, a promoter activity assay indicated that DH5 α harbouring pJZL, in which P_{luxS} directs the expression of a promoterless luciferase gene, exhibited luciferase activity that was approximately ninefold higher in the absence than in the presence of cAMP, suggesting that the *E. coli* CRP repressed transcription from P_{luxS} . Consistent with this conclusion, *in vitro* EMSA demonstrated that the purified

recombinant *E. coli* CRP could bind specifically to the DNA fragment containing P_{luxS} (Fig. 4). Sequence inspection revealed a potential CRP-binding site that partially matches the consensus CRP-binding sequence, TGTGAN₆TCACA, 13 bp downstream of P_{luxS} (Fig. 2). Taken together, these results indicated that glucose upregulates *luxS_{Et}* expression by inactivating CRP, which represses transcription from P_{luxS} .

Interference with *luxS_{Et}* expression has pleiotropic effects

Effect on AI-2 activity. Given that production of *LuxS_{Et}* is apparently regulated at the expression level, it was interesting to examine the effect of interrupting the regulated synthesis of *LuxS_{Et}*. With this aim, several attempts were made to construct a TX1 variant with inactivated *luxS_{Et}* but all were unsuccessful; we therefore adopted the strategy of antisense RNA interference. For this purpose, the conjugative plasmid pJR18, in which the *luxS_{Et}* antisense RNA (corresponding to the region between positions 149 and -65 relative to the translational start) is constitutively transcribed, was introduced into TX1 by conjugation. Production of the antisense RNA in the transconjugant TX1/pJR18 was confirmed by real-time RT-PCR (data not shown). Real-time RT-PCR analysis indicated that transcription of *luxS_{Et}* in TX1/pJR18 grown in LB medium at 28 °C was ~ 80 % lower than that in TX1/pJRA grown under the same conditions, suggesting that the antisense RNA exerted a negative effect either on the transcription or on the stability of *luxS_{Et}* mRNA. AI-2 assay showed that the AI-2 activity in TX1/pJR18 was 66 % lower than that in TX1/pJRA. In contrast the presence of the antisense RNA of *acrA*, which encodes the acriflavine-resistance protein A of *Ed. tarda* (J. Hou & L. Sun, unpublished) failed to have any effect on AI-2 activity or *luxS_{Et}* expression (data not shown). Therefore the reduced *luxS_{Et}* expression and AI-2 activity observed with TX1/pJR18 were the specific effect of attenuated *luxS_{Et}* expression.

Fig. 4. Specific binding of CRP to the promoter region of *luxS_{Et}*. EMSA was performed in CRP binding buffer containing the carboxyfluorescein-labelled DNA fragment harbouring P_{luxS} and purified recombinant *E. coli* CRP in the presence or absence of cAMP. After the reaction, the samples were run on a nondenaturing 8 % polyacrylamide gel. The DNA in lanes 5 and 6 served as controls.

Effect on growth. Growth pattern studies showed that, compared with TX1/pJRA, TX1/pJR18 displayed a slower generation time (*g*) and lower maximum cell densities under all the conditions examined, which included growth in LB medium at 28 °C, under iron depletion caused by the iron chelator 2,2'-dipyridyl, in LB medium at 37 °C, and in 0.6 M NaCl (2216E medium) at 28 °C (Fig. 5); under these conditions, the differences in *g* between TX1/pJRA and TX1/pJR18 were 8.6, 10.3, 30.4 and 9.5 %, respectively, while the differences in maximum cell density between TX1/pJRA and TX1/pJR18 were 12.1, 25.3, 78.9 and 44 %, respectively. *LuxS* is known to function in two cellular aspects, one in cell-cell signalling (via AI-2) and the other

Fig. 5. Growth patterns of *Ed. tarda* TX1/pJRA and TX1/pJR18 under various conditions. (a) Cells were cultured at 28 °C in LB medium with or without 100 µM 2,2'-dipyridyl (DP). (b) Cells were cultured in LB at 37 °C or in 2216E medium at 28 °C. *g*, mean generation time.

in the activated methyl cycle; complementation of a physiological defect by exogenous AI-2 can be an indicator that the particular defect is due to impairment in the former function of LuxS. In the case of TX1/pJR18, addition of exogenous AI-2 in the form of AI-2-containing culture supernatant of TX1 failed to rescue the growth defects (data not shown), suggesting that these defects are probably not the result of changes in AI-2-mediated signalling process but more likely that of impairment in central metabolism.

Effect on pathogenicity. To determine whether reduced expression of *luxS_{Et}* had any effect on bacterial pathogenicity, an animal infection study was carried out to examine the virulence potential of TX1/pJRA and TX1/pJR18. For this purpose Japanese flounders were injected i.p. with one of the two strains and monitored for cumulative mortality. Administration of TX1/pJRA at a dose of 4×10^6 c.f.u. led to 95 % mortality within the monitored period whereas administration of the same dose of TX1/pJR18 resulted in 70 % accumulated mortality, which was significantly ($P < 0.01$ %) lower than that caused by TX1/pJRA. In both infections, the bacteria were detectable in the blood and organs (kidney and liver) as early as 4 h and 12 h post-infection, respectively, but the amounts of TX1/pJR18 in the blood and organs were significantly lower than that of TX1/pJRA during the entire infection period (Fig. 6). To investigate whether the difference in tissue dissemination between TX1/pJR18 and TX1/pJRA was due to selective loss of pJRA under survival pressures, the organs (kidney and liver) and the blood were taken from the fish infected with TX1/pJR18 and TX1/pJRA at 1, 2, and 3 days post-infection; the organs were homogenized in PBS, and the homogenized organs and the blood were plated on LB agar plates supplemented with tetracycline alone (for the selection of TX1, TX1/pJRA and TX1/pJR18) and on LB plates supplemented with tetracycline and ampicillin (for the selection of TX1/pJRA and TX1/pJR18). With the blood and organs derived from TX1/pJR18-infected fish, the numbers of TX1/pJR18 that appeared on the tetracycline plus ampicillin plates were 12.8–14.4 % lower than those of

TX1 that appeared on the tetracycline plates; PCR analysis using TX1- and pJR18-specific primers indicated that 85.7 % (60/70) of the TX1 colonies on the tetracycline plates were TX1/pJR18. With the blood and organs derived from the fish infected with TX1/pJRA, the numbers of TX1/pJRA that emerged on the tetracycline plus ampicillin plates were 8.7–10.2 % lower than those of TX1 that emerged on the tetracycline plates; 90 % (63/70) of the TX1 colonies on the tetracycline plates were verified to be TX1/pJRA. Hence, the plasmid loss rate of TX1/pJR18 was ~5.7 % higher than that of TX1/pJRA within the host environment; however, this difference in plasmid stability was apparently too low to account for the 10- to 100-fold difference (Fig. 6) in the amount of TX1/pJR18 and TX1/pJRA recovered from the blood and organs of the infected fish.

Taken together, these results demonstrated that interference with *luxS_{Et}* expression attenuates the virulence and vitiates the infection/survival ability of TX1.

Effect on the expression of virulence-associated factors. To find out the potential genetic mechanisms underlying the attenuated virulence observed with TX1/pJR18, we examined the production/expression in TX1/pJR18 of known virulence elements: the type III secretion system (TTSS), type VI secretion system, Eth haemolysin system and biofilm formation (Hirono *et al.*, 1997; Tan *et al.*, 2005; Zheng & Leung, 2007). The TTSS genes are located in two chromosomal regions, region 1 and region 2; real-time RT-PCR analysis demonstrated that *luxS_{Et}* antisense RNA had no effect on the expression of *eseB* and *eseD*, which are in region 1, but reduced the expression of *esrA* and *orf26* (Fig. 7a), which are in region 2. To determine whether *luxS_{Et}* antisense RNA had any effect on biofilm production, TX1/pJRA and TX1/pJR18 were cultured to OD₆₀₀ 0.8 and placed into a 96-well polystyrene plate; after incubation at 28 °C for 24 h, the development of biofilm was examined. Biofilm production was reduced threefold in TX1/pJR18 compared with that in TX1/pJRA (Fig. 7b). Addition of AI-2-containing cell-free culture supernatant of TX1 restored both biofilm

Fig. 6. Bacterial dissemination in the kidney (a) and blood (b) of infected fish. Flounders were i.p. injected with *Ed. tarda* TX1/pJR18 or TX1/pJRA and the blood and kidneys of at least three fish were taken at various time points post-infection. The homogenized organs and the blood were plated on selective LB plates and the number of plasmid-bearing TX1 was counted. Data are presented as the means $\pm$ SEM. **, $P < 0.01$.

Fig. 7. Comparison of the expression of TTSS genes (a) and biofilm formation (b) in *Ed. tarda* TX1/pJRA and TX1/pJR18. (a) Real-time RT-PCR was performed using total RNA extracted from cells grown to OD₆₀₀ 0.8 in LB medium supplemented with or without AI-2-containing cell-free culture fluids of strain TX1 (10%). 16S rRNA was used as a control. For each gene, the mRNA level of TX1/pJRA was set as 1. (b) Biofilm formation on a polystyrene surface was determined for TX1/pJRA and TX1/pJR18 in the presence or absence of AI-2 supplemented as above. Data are presented as the means $\pm$ SEM. **, $P < 0.01$.

production and *orf26/esrA* expression in TX1/pJR18 to a level approaching that in TX1/pJRA but had no effect on the expression of *eseB* and *eseD* (Fig. 7). These results suggested that LuxS_{Et} modulates the expression of *orf26/esrA* and biofilm development probably through the action of AI-2, which implies the existence in TX1 of a functional LuxS/AI-2-mediated signalling system. To determine whether there was any difference in *orf26/esrA* expression between TX1/pJR18 and TX1/pJRA during infection, real-time RT PCR was carried out to analyse the expression of *orf26/esrA* using RNA extracted from the spleens and livers taken 24 h after the fish were infected with TX1/pJR18 and TX1/pJRA. The results showed that the expression levels of *orf26* and *esrA* were, respectively, 5.7- and 4-fold lower in TX1/pJR18-infected fish than those in TX1/pJRA-infected fish (data not shown); hence interference with *luxS_{Et}* expression had a significant effect on the expression of *orf26* and *esrA* during infection.

Real-time RT PCR analysis of the expression of the haemolysin-encoding genes *ethA* and *ethB* and the type VI secretion system genes *evpA* and *evpB* showed that *luxS_{Et}* antisense RNA had no significant effect on the expression of these genes (data not shown).

DISCUSSION

Surette & Bassler (1999) observed that in *Salmonella typhimurium* AI-2 production is affected by environmental

factors such as glucose and pH, which boost the AI-2 level. In our study we found that in *Ed. tarda* TX1 both AI-2 activity and *luxS_{Et}* expression were influenced positively by glucose and negatively by high temperature. Studies by Wang *et al.* (2005) revealed that in *E. coli* AI-2 production and *luxS* expression are subject to catabolite repression through the cAMP–CRP complex, which indirectly inhibits the expression of *luxS* and directly stimulates the expression of *lsr*, which encodes an ATP-binding cassette transporter that functions as an AI-2 uptake system (Taga *et al.*, 2001, 2003; Xavier & Bassler, 2005). In the case of TX1, glucose modulated *luxS_{Et}* expression via cAMP–CRP, which directly repressed *luxS_{Et}* expression, probably by blocking the formation of the DNA–RNA polymerase complex, considering the location of the putative CRP-binding site that is downstream of P_{luxS}. Although Lsr-like transporting systems have not yet been discovered in *Ed. tarda*, it is possible that in *Ed. tarda* TX1 the glucose-associated increase of AI-2 activity is the combined result of both enhanced production, due to augmented expression of *luxS_{Et}*, and reduced clearance of the signalling molecule.

In our study we observed a rough correlation between AI-2 activity and *luxS_{Et}* expression, which suggested that in strain TX1 production of AI-2 activity is at least in part controlled at the level of *luxS_{Et}* expression. Interference with the regulated expression of *luxS_{Et}* and AI-2 synthesis appeared to have severe consequences upon certain physiological processes that manifested, in one form, as growth defects. The failure of the growth defects to be complemented by exogenous AI-2 rules out, to a large extent, the involvement of AI-2-mediated signal transduction. As one of the enzymes participating in the activated methyl cycle, LuxS plays an important role in cellular metabolism (Doherty *et al.*, 2006; Vendeville *et al.*, 2005; Winzer *et al.*, 2002a); hence interruption of *luxS* expression has a direct effect on fundamental cellular processes (Kendall *et al.*, 2007), which probably accounts for the growth deficiencies observed with TX1/pJR18 under various conditions.

It is well documented that LuxS is involved in the regulation of virulence development in diverse bacteria (Coulthurst *et al.*, 2004, 2007; Day & Maurelli, 2001; Joyce *et al.*, 2004). In *E. coli*, *V. harveyi* and *Streptococcus pyogenes* LuxS has been associated with the production of virulence factors such as the TTSS, extracellular proteases and biofilm development (Henke & Bassler, 2004a, b; Herzberg *et al.*, 2006; Li *et al.*, 2007; Lyon *et al.*, 2001; Sircili *et al.*, 2004). In TX1 we found that interference with *luxS_{Et}* expression affected biofilm production and the expression of TTSS-encoding genes located in DNA region 2. Given the fact that *luxS_{Et}* expression was not entirely blocked off but only reduced by the antisense RNA, the null effect observed with *eseB/eseD*, *evpA/evpB* and *ethA/ethB* cannot rule out the possibility that these genes are subject to LuxS_{Et} regulation but to a lesser degree than that observed with *orf26/esrA* and hence undetectable under our

experimental conditions. Since both the TTSS and biofilm formation have been related to bacterial pathogenicity, the reduced production of these elements may have contributed to the attenuated virulence observed with TX1/pJR18. This hypothesis is consistent with the observation that interference with *luxS_{Et}* expression significantly altered the expression of *orf26* and *esrA* during infection. In addition, the effect of *luxS_{Et}* antisense RNA on growth may play a part in mitigating the bacterial virulence of TX1/pJR18.

In conclusion, our study has demonstrated that in *Ed. tarda* TX1 AI-2 activity correlates to a certain degree with the expression of *luxS_{Et}*, which is in turn regulated by growth phase and growth conditions. Our results favour the notion that LuxS_{Et} plays a dual role, one in cellular metabolism and the other in AI-2-mediated signalling; interruption of the first role of LuxS_{Et} leads to a growth defect whereas interruption of the second leads to aberrations in the development of certain biological characteristics that are required for full bacterial virulence.

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