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The *hanR/hanI* quorum-sensing system of *Halomonas anticariensis*, a moderately halophilic bacterium

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Quorum sensing is a cell density-dependent gene expression mechanism found in many Gram-negative bacteria which involves the production of signal molecules such as *N*-acylhomoserine lactones (AHLs). One significant group of micro-organisms in which quorum sensing has not been previously studied, however, are the moderate halophiles. We describe here the results of our studies of the quorum-sensing system in *Halomonas anticariensis* FP35^T, which is composed of *luxR/luxI* homologues: *hanR* (the putative transcriptional regulator gene) and *hanI* (the autoinducer synthase gene). To understand how the *hanR/hanI* system is organized and regulated we conducted RT-PCR and quantitative real-time PCR assays. Transcriptional analysis indicated that the *hanR* and *hanI* genes are on the same transcript and that their transcription is growth phase-dependent. HanI seems to be the only autoinducer synthase responsible for the synthesis of AHLs by the bacterium, since the inactivation of *hanI* resulted in the complete loss of its AHLs. We also found that the *hanI* gene appears to be transcribed from its own promoter and that its expression does not depend upon HanR. This finding was supported by the fact that the FP35*hanR* mutant showed AHL-producing activity and *hanI* expression similar to that of the wild-type strain, the latter being measured by RT-PCR. Moreover, *hanR* is expressed from its own promoter and appears to be independent of the AHL signalling molecules produced by HanI.

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INTRODUCTION

Over the past few years it has become increasingly evident that numerous bacteria can establish cell-to-cell communication within their communities by producing small signal molecules that either diffuse freely across the cell membranes or are actively transported out of the cell. These signals, known as autoinducers, control gene expression in response to bacterial cell density in a process called quorum sensing (reviewed by González & Marketon, 2003; Ng & Bassler, 2009; Parker & Sperandio, 2009).

The most thoroughly characterized Gram-negative, bacterial, intraspecific autoinducers are *N*-acylhomoserine lactones (AHLs), which have been reported to be synthesized by a member of the LuxI protein family in over 70 genera belonging to the phylum Proteobacteria. AHL molecules

differ in the length and substitution of their respective acyl side chains, conferring upon them signal specificity. As the density of the population increases AHL molecules accumulate in the growth medium, and on reaching a critical concentration threshold they bind and activate an AHL receptor protein belonging to the LuxR family of transcriptional regulators. The activated LuxR/AHL complex then binds to a specific palindromic sequence on the DNA, known as the 'lux box', located upstream of the genes regulated by quorum sensing. This results in the activation or repression of target genes, including in many cases the activation of the autoinducer synthase, which leads in turn to the production of more AHLs and consequently a positive feedback loop (Eberhard *et al.*, 1991; Fuqua *et al.*, 1994).

This phenomenon has usually been found in bacteria that live in pathogenic or symbiotic associations in which quorum-sensing systems regulate genes involved in the expression of virulence factors and exoenzymes (de Kievit & Iglewski, 2000; Song *et al.*, 2005; von Bodman *et al.*, 2003), conjugal DNA transfer and plasmid copy number control (Farrand, 1998; Fuqua *et al.*, 2001, 1994; Marketon & González, 2002), production of and susceptibility to antibiotics (Horng *et al.*, 2002; McClean *et al.*, 1997; Pumbwe *et al.*, 2008; Van Houdt *et al.*, 2007), biofilm formation (Davies *et al.*, 1998; McNab *et al.*, 2003; Niu

Abbreviations: AHL, *N*-acylhomoserine lactone; C₄-HL, *N*-butanoyl homoserine lactone; C₆-HL, *N*-hexanoyl homoserine lactone; C₈-HL, *N*-octanoyl homoserine lactone; C₁₂-HL, *N*-dodecanoyl homoserine lactone; 3-oxo-C₆-HL, *N*-(β-ketocaproyl)-L-homoserine lactone; RT-qPCR, reverse transcription quantitative real-time PCR.

The GenBank/EMBL/DDBJ accession number for the *hanR-hanI* DNA sequence of *H. anticariensis* is HM236058.

Three supplementary figures and two supplementary tables are available with the online version of this paper.

et al., 2008) and exopolysaccharide production (Marketon *et al.*, 2003; von Bodman *et al.*, 1998).

In addition to AHLs, other quorum-sensing autoinducer molecules recently identified in Gram-negative bacteria are autoinducer 2 (AI-2) (Chen *et al.*, 2002), which has been described as a global signal molecule for interspecific communication, 2-alkyl-4-quinolones (AQs), such as PQS in *Pseudomonas* (Diggle *et al.*, 2007), long-chain fatty acids, such as the 'diffusible signal factor' (DSF) in *Xanthomonas campestris* (Wang *et al.*, 2004), *p*-coumaroyl-HSL in *Rhodospseudomonas palustris* (Schaefer *et al.*, 2008) and others (González & Keshavan, 2006; Ng & Bassler, 2009).

It has been reported that in complex environments bacterial signalling does not accumulate at a constant rate (Boyer & Wisniewski-Dyé, 2009). The diffusion and perception of AHLs in and around cells can be influenced by abiotic environmental factors such as pH, temperature and medium composition, and by other members of the bacterial community in a process known as 'quorum quenching' (Defoirdt *et al.*, 2008; Taga & Bassler, 2003). A variety of bacterial biosensor strains have been constructed to detect autoinducer molecules (Llamas *et al.*, 2004; McClean *et al.*, 1997; Shaw *et al.*, 1997; Winson *et al.*, 1998). These strains do not produce any AHL signal molecules but can detect exogenous AHLs by the activation of a reporter gene such as *lacZ* or *lux* or, in *Chromobacterium violaceum*, by the production or inhibition of a purple pigment. The use of these biosensors in a previous study led us to observe that the exopolysaccharide-producing *Halomonas anticariensis* FP35^T synthesizes quorum-sensing molecules in a growth phase-dependent manner and that its maximum activity is reached by the end of the exponential phase. In addition, using electrospray ionization MS (ESI MS/MS), we have identified some of the AHL molecular structures in this bacterium, such as *N*-butanoyl homoserine lactone (C₄-HL), *N*-hexanoyl homoserine lactone (C₆-HL), *N*-octanoyl homoserine lactone (C₈-HL) and *N*-dodecanoyl homoserine lactone (C₁₂-HL) (Llamas *et al.*, 2005).

Cell density-dependent gene regulation has not been studied to date in extreme environments such as those inhabited by *Halomonas* species. The genus *Halomonas* currently comprises more than 70 species of halophilic bacteria, most of which have been isolated from saline environments such as salterns, saline soils, seawater and marshes (Euzéby, 2010). Its members are extremely versatile from the ecological, physiological and metabolic point of view. The interest in *Halomonas* species has centred on their ability to degrade aromatic compounds and produce compatible solutes, halophilic enzymes and exopolysaccharides with potential applications in biotechnology (Sutherland, 2002).

In this study we have genetically characterized the quorum-sensing system involved in the production of AHLs in *H. anticariensis* FP35^T, which is composed of a putative transcriptional regulator HanR (LuxR homologue) and an AHL synthase HanI (LuxI homologue). Gene expression

analysis demonstrates that the *hanR/hanI* genes are organized in the same operon and that their expression is cell density-dependent. The expression of *hanR* appears to be independent of the AHL signalling molecules produced by HanI, an unusual characteristic compared with other LuxRI-like systems already characterized (González & Marketon, 2003).

METHODS

Bacterial strains, plasmids, media and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *H. anticariensis* strains were routinely cultured at 32 °C in SWYE (Nieto *et al.*, 1989) or MY medium (Moraine & Rogovin, 1966) modified with a balanced mixture of sea salts (Rodríguez-Valera *et al.*, 1981). *Escherichia coli* strains were grown at 37 °C in LB medium (Sambrook & Russell, 2001). Antibiotics were added at the following final concentrations: rifampicin, 50 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; gentamicin, 25 µg ml⁻¹; tetracycline, 15 µg ml⁻¹; and ampicillin, 100 µg ml⁻¹. *Agrobacterium tumefaciens* NTL4 (pZLR4) was cultured at 30 °C in LB medium supplemented with 2.5 mM CaCl₂ and 2.5 mM MgSO₄ (LB/MC) and in MGM minimal medium (per litre: 11 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 mg biotin, 27.8 mg CaCl₂, 246 mg MgSO₄) containing 50 µg gentamicin ml⁻¹. *C. violaceum* CV026 was grown at 30 °C in LB medium.

Transposon mutagenesis and isolation of the FP35-111 (FP35::hanR::mini-Tn5 Km2) mutant. Transposon mutagenesis was carried out via conjugation by biparental mating, as described in a previous publication (Llamas *et al.*, 2000). The donor strain was *E. coli* S17-1 *λ*pir, harbouring the suicide vector pUT mini-Tn5 Km2 (de Lorenzo *et al.*, 1990). The recipient strain was *H. anticariensis* FP35-R, a spontaneous rifampicin-resistant mutant of *H. anticariensis* FP35^T. The transconjugants were isolated on SWYE medium containing 2% (w/v) salts, rifampicin (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹). Selected clones were replicated onto both the same isolating medium and trypticase soy agar (TSA) (per litre: 15 g trypticase peptone, 5 g phytone peptones, 5 g NaCl, 15 g agar) and incubated overnight at 32 °C. The halophilic clones that did not grow in TSA medium due to its low salt content were then streaked onto LB/MC or MGM media, where the indicator strains [*C. violaceum* CV026 or *A. tumefaciens* NTL4 (pZLR4)] had been previously streaked in order to isolate AHL-deficient strains. They were incubated overnight at 32 °C to allow the indicator organisms to grow and surround the clone growths with either purple or blue haloes.

Cloning of the *hanRI* genes. To isolate the DNA fragment carrying the mini-Tn5 insertion, which encodes the kanamycin-resistance gene, genomic DNA from *H. anticariensis* FP35-111 was completely digested with enzymes that do not cleave within the minitransposon region. DNA fragments were separated on an agarose gel and transferred onto a nylon filter by standard techniques (Sambrook & Russell, 2001). Fragments containing the mini-Tn5 Km2 insertion were analysed by Southern hybridization using a digoxigenin DNA labelling and detection kit (Boehringer Mannheim) as described in previous publications (Llamas *et al.*, 1997, 2003). Chromosomal DNA fragments digested with *Pst*I were selected and ligated into pGEM-T to create pP111. The cloned fragments were sequenced directly by primer walking using a BigDye Terminator Cycle Sequencing kit in an ABI 3100 DNA sequencer (Applied Biosystems). The genomic sequence located upstream of the known nucleotide sequence was obtained by inverse PCR (Ochman *et al.*, 1988; Llamas *et al.*, 2003). One plasmid, pBFP35, was isolated and sequenced. The DNA

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Rif^r, rifampicin resistance; Tc^r, tetracycline resistance.

Strain or plasmid	Genotype or description	Reference or source
<i>H. anticariensis</i> strains:		
FP35 ^T (=CECT 5854 ^T)	Wild-type isolate, mucoid	Martínez-Cánovas <i>et al.</i> (2004)
FP35-R	Spontaneous rifampicin-resistant mutant of FP35, Rif ^r	This work
FP35-11I	FP35-R containing <i>hanR</i> ::mini-Tn5 Km2, Rif ^r , Km ^r	This work
FP35 <i>hanI</i>	FP35-R containing <i>hanI</i> :: <i>lacZ</i> , Rif ^r , Km ^r	This work
FP35 <i>hanR</i>	FP35-R containing <i>hanR</i> :: <i>lacZ</i> , Rif ^r , Km ^r	This work
FP35(pMP220)	FP35-R containing pMP220, Rif ^r , Tc ^r	This work
FP35(pMP-phanR)	FP35-R containing pMP-phanR, Rif ^r , Tc ^r	This work
FP35(pMP-phanI)	FP35-R containing pMP-phanR, Rif ^r , Tc ^r	This work
FP35-11I(pJN-HanR)	FP35-R containing <i>hanR</i> ::mini-Tn5 Km2 and pJN-HanR, Rif ^r , Km ^r , Gm ^r	This work
FP35 <i>hanI</i> (pJN-HanI)	FP35-R containing <i>hanI</i> :: <i>lacZ</i> and pJN-HanI, Rif ^r , Km ^r , Gm ^r	This work
<i>A. tumefaciens</i> NTL4(pZLR4)	NT1 derivative carrying a <i>traG</i> :: <i>lacZ</i> reporter fusion, Gm ^r	Shaw <i>et al.</i> (1997)
<i>C. violaceum</i> CV026	CV017 derivative containing <i>cviI</i> ::Tn5 <i>xylE</i> , Km ^r	McClellan <i>et al.</i> (1997)
<i>E. coli</i> strains:		
DH5 α	F ⁻ ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZY-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁻) <i>supE44</i> λ ⁻ <i>thi-1 girA96 relA1</i>	Laboratory collection
S17-1 λ <i>pir</i>	RK2 <i>tra</i> regulon, <i>pir</i> , host for <i>pir</i> -dependent plasmids	Miller & Mekalanos (1988)
Plasmids:		
pGEM-T	High-copy-number cloning vector, Ap ^r , <i>bla</i> , <i>lacZ</i>	Promega
pUT mini-Tn5 Km2	Ap ^r , Km ^r , <i>ori</i> R6K, <i>oriT</i> RP4	de Lorenzo <i>et al.</i> (1990)
pP11I	pGEM-T with a 5 kb <i>PstI</i> fragment carrying mini-Tn5 insertion, Ap ^r , Km ^r	This work
pBFP35	pGEM-T with a 1.6 kb inverse PCR product amplified from a 2.2 kb fragment from strain FP35 genome, Ap ^r	This work
pVIK112	<i>lacZY</i> for disruption and transcriptional fusion, Km ^r	Kalogeraki & Winans (1997)
pVIKHanI	pVIK112 containing an internal <i>hanI</i> fragment, transcriptional fusion, Km ^r	This work
pVIKHanR	pVIK112 containing an internal <i>hanR</i> fragment, transcriptional fusion, Km ^r	This work
pMP220	Promoter-probe vector, ' <i>lacZ</i> ; IncP; Tc ^r	Spaink <i>et al.</i> (1987)
pMP-phanI	pMP220 containing <i>hanI</i> promoter	This work
pMP-phanR	pMP220 containing <i>hanR</i> promoter	This work
pJN105	<i>araC</i> -P _{BAD} cassette cloned in pBBR1MCS5	Newman & Fuqua (1999)
pJN-HanI	pJN105 containing intact <i>hanI</i> , Gm ^r	This work
pJN-HanR	pJN105 containing intact <i>hanR</i> , Gm ^r	This work

sequences thus obtained were analysed using a BLAST search of the GenBank database (National Center for Biotechnology Information; NCBI; <http://www.ncbi.nlm.nih.gov>). Promoter regions were predicted with the BPROM software (Softberry; <http://linux1.softberry.com/berry.phtml>). The conserved domains of the LuxR-type transcriptional regulator were identified using the SMART program (Letunic *et al.*, 2006).

DNA manipulations. All molecular techniques were performed using standard protocols (Sambrook & Russell, 2001). The primers used in this study are listed in Supplementary Table S1.

Construction of the *hanR* and *hanI* gene mutations. An internal fragment lacking both the 5' and 3' ends of the *hanR* and *hanI* genes was cloned separately into the suicide plasmid pVIK112, containing the promoterless *lacZ* gene. Chromosomal gene disruptions were then effected in a single step by Campbell-type integration of the entire plasmid by homologous recombination (Kalogeraki & Winans, 1997).

Internal segments of 301 and 356 bp of the *hanR* and *hanI* genes, respectively, were amplified from *H. anticariensis* FP35^T chromosomal DNA by using the primers hanRsub-*EcoRI*-F and hanRsub-*XbaI*-R for *hanR*, and hanIsub-*EcoRI*-F and hanIsub-*XbaI*-R for *hanI*,

which contain *EcoRI* and *XbaI* restriction sites (underlined) at their respective 5' ends (Supplementary Table S1). PCR entailed 30 cycles of 30 s at 95 °C, 30 s at 66 °C and 30 s at 72 °C. The PCR fragments were purified, cloned into pGEM-T, and digested with *EcoRI* and *XbaI*. The purified fragments were then cloned into the suicide plasmid pVIK112. The resulting plasmids, pVIKHanR and pVIKHanI, were subsequently transformed separately into *E. coli* S17-1 λ *pir* and transferred into a rifampicin-resistant FP35 derivative (FP35-R) by biparental mating, where the promoterless reporter *lacZ* gene simultaneously disrupted and created fusions to the *hanR* and *hanI* genes by single recombination. The *H. anticariensis* transconjugants, FP35*hanI* and FP35*hanR*, were selected by plating them on SWYE medium containing 2% (w/v) salts, rifampicin (50 μ g ml⁻¹) and kanamycin (50 μ g ml⁻¹) (Kalogeraki & Winans, 1997; Llamas *et al.*, 2004) (Table 1).

Cloning of the *hanR* and *hanI* genes in the expression vector pJN105. The ORFs of *hanR* and *hanI* were amplified from *H. anticariensis* FP35^T chromosomal DNA by using the primers hanR-*EcoRI*-F and hanR-*XbaI*-R, and hanI-*EcoRI*-F and hanI-*XbaI*-R, respectively, which contain *EcoRI* and *XbaI* restriction sites at their respective 5' ends (Supplementary Table S1). PCR entailed 30 cycles

of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C. The PCR fragment was purified, digested with *EcoRI* and *XbaI*, and cloned into the broad-host-range expression vector pJN105, which carries the L-arabinose-inducible *E. coli araBAD* promoter and the *araC* regulator (*araC*-P_{BAD}) (Newman & Fuqua, 1999) to create pJN-HanR and pJN-HanI. The pJN-HanR and pJN-HanI plasmids were then transformed separately into *E. coli* S17-1 λ pir and transferred by biparental mating into the AHL-deficient strains FP35-11I and FP35hanI, respectively, to be used in complementation analysis. To confirm whether AHL production was restored in strains FP35-11I (pJN-HanR) and FP35hanI (pJN-HanI), signal molecules were extracted and analysed following the technique described in previous studies (Llamas *et al.*, 2004, 2005; Marketon *et al.*, 2002). All constructs were confirmed by PCR.

RNA extraction and RT-PCR assays. Total bacterial RNA was isolated using the RNA II Nucleospin kit (Macherey-Nagel), followed by rigorous treatment with Turbo DNA-free RNase (Ambion) according to the manufacturer's protocol. All RNA samples were visualized on the gel and the concentrations were measured with a NanoDrop ND-2000c spectrophotometer (Thermo Scientific). RT-PCR was conducted with 0.05–0.5 μ g total RNA at a final volume of 50 μ l using the SuperScript III One-Step RT-PCR System with PlatinumTaq according to the manufacturer's instructions (Invitrogen). cDNA was synthesized at 55 °C for 30 min. Denaturation was performed for 2 min at 94 °C, followed by 35 cycles of PCR, as suggested by the manufacturer. A final elongation step was conducted at 68 °C for 7 min. The annealing temperature was calculated for each reaction on the basis of the melting temperatures of the pairs of primers used (Supplementary Table S1). Positive and negative controls were included in all the assays. 16S rRNA was used as a control for normalization. RT-PCR products were visualized by electrophoresis.

Reverse transcription quantitative real-time PCR analysis (RT-qPCR). Total RNA from bacterial cultures growing at the early exponential and stationary phases (OD₆₀₀ values of 0.4 and 1.4, respectively) was extracted as described above. All RNA samples were prepared from two independent cultures. RNA preparations (1 μ g) treated with Turbo DNA-free RNase (Ambion) were reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad). RT-qPCR was done with an iCycler iQ system (Bio-Rad). Each reaction mixture contained 1 μ l of the target cDNA or a dilution (1:10 000, for amplification of the 16S rRNA gene), 100 nM of each of the forward and reverse primers, 10 μ l 2 \times iQ SYBR Green Supermix (Bio-Rad) and nuclease-free water to a final volume of 20 μ l. All reactions were repeated in triplicate. PCRs were also carried out with the RNA samples untreated with reverse transcriptase to confirm the absence of contaminating genomic DNA. The oligonucleotide sequences used for RT-qPCR analysis are listed in Supplementary Table S1. The amplification conditions consisted of an initial 3 min denaturation step at 95 °C, followed by 40 cycles each of denaturation at 95 °C for 10 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. Negative controls without cDNA for each primer set were included in each run. At the end of the real-time PCR cycles, a melting curve was generated and analysed to confirm product specificity. For all genes, melting-curve analysis showed clear melting peaks without non-specific products or artefacts. Standard curves were obtained using recombinant plasmids containing a fragment of the gene of interest. The relative expression of each gene was normalized to that of 16S rRNA and evaluated during the early exponential phase versus the stationary phase using the Relative Expression Software Tool (REST2009) (Pfaffl *et al.*, 2002).

Analysis of *hanR* and *hanI* promoter activity. The promoter regions of the *hanR* and *hanI* genes (*phanR* and *phanI* respectively) were amplified by PCR with primers incorporating *Bgl*III and *Xba*I linkers for *phanR*, and *Eco*RI and *Xba*I linkers for *phanI*, respectively

(Supplementary Table S1). The promoter region of each gene was fused to the *lacZ* gene in the promoterless low-copy-number pMP220 vector (Spaink *et al.*, 1987). Upon amplification, DNA was cloned into the pGEM-T vector, digested with the appropriate restriction enzymes, and ligated to pMP220 for the construction of pMP-*phanR* and pMP-*phanI*. The constructions were confirmed by DNA sequencing. The pMP220, pMP-*phanR* and pMP-*phanI* plasmids were then transformed separately into *E. coli* S17-1 λ pir and transferred by biparental mating into *H. anticariensis* FP35-R (Table 1). Promoter activity was analysed by measuring β -galactosidase activity (Miller, 1972).

Extraction, detection and TLC analysis of crude AHL extracts. AHL molecules were extracted following the technique described in our previous studies (Llamas *et al.*, 2004; Marketon *et al.*, 2002). To detect AHLs, an overnight culture of the AHL indicator strain *C. violaceum* CV026 was diluted 1:100 in 5 ml LB medium and poured onto LB agar. Once the plates were dried, paper disks 5 mm in diameter were placed on an agar plate and the AHL samples were applied. The assay plates were incubated overnight at 32 °C and the appearance of pigment around the filter was determined. To characterize the AHLs, the samples were subjected to analytical and preparative TLC. AHL samples and standards were spotted onto a TLC plate and developed with 70% (v/v) methanol in water. The plate was air-dried and overlaid with top agar containing the *A. tumefaciens* NTL4 (pZLR4) indicator strain before being incubated at 32 °C (Llamas *et al.*, 2005).

AHL standards were obtained from Sigma [*N*-(β -ketocaproyl)-L-homoserine lactone (3-oxo-C₆-HL)] and Fluka (C₆-HL and C₈-HL).

RESULTS AND DISCUSSION

Isolation and characterization of the AHL-deficient strain FP35-11I

In a previous paper we reported that the exopolysaccharide-producing *H. anticariensis* FP35^T synthesizes at least four AHLs (C₄-HL, C₆-HL, C₈-HL and C₁₂-HL) (Llamas *et al.*, 2005). In an effort to identify the genes involved in their production, one of the four partially AHL-deficient *H. anticariensis* clones obtained by transposon mutagenesis, the FP35-11I mutant, was chosen for this study because it activated the two indicator strains to a lesser extent than did the wild-type strain. To further confirm the lower AHL production exhibited by this mutant, 5 ml culture extracts from both the *H. anticariensis* wild-type and FP35-11I mutant strains were analysed on an agar plate and by TLC. Analysis with the CV026 and NTL4 (pZLR4) indicator organisms revealed that the FP35-11I mutant (Fig. 1a, panel 2 and b, lane 2) produced lower levels of AHLs than the wild-type strain (Fig. 1a, panel 1 and b, lane 1).

Cloning and sequencing of *luxR* and *luxI* homologues from *H. anticariensis* FP35^T

The nucleotide sequences of the chromosomal DNA in the two recombinant plasmids, pP11I and pBFP35 (see Methods), revealed the presence of two complete ORFs (Fig. 2a). The transposon insertion site within the FP35-11I genome was located after base pair 394 from the translation

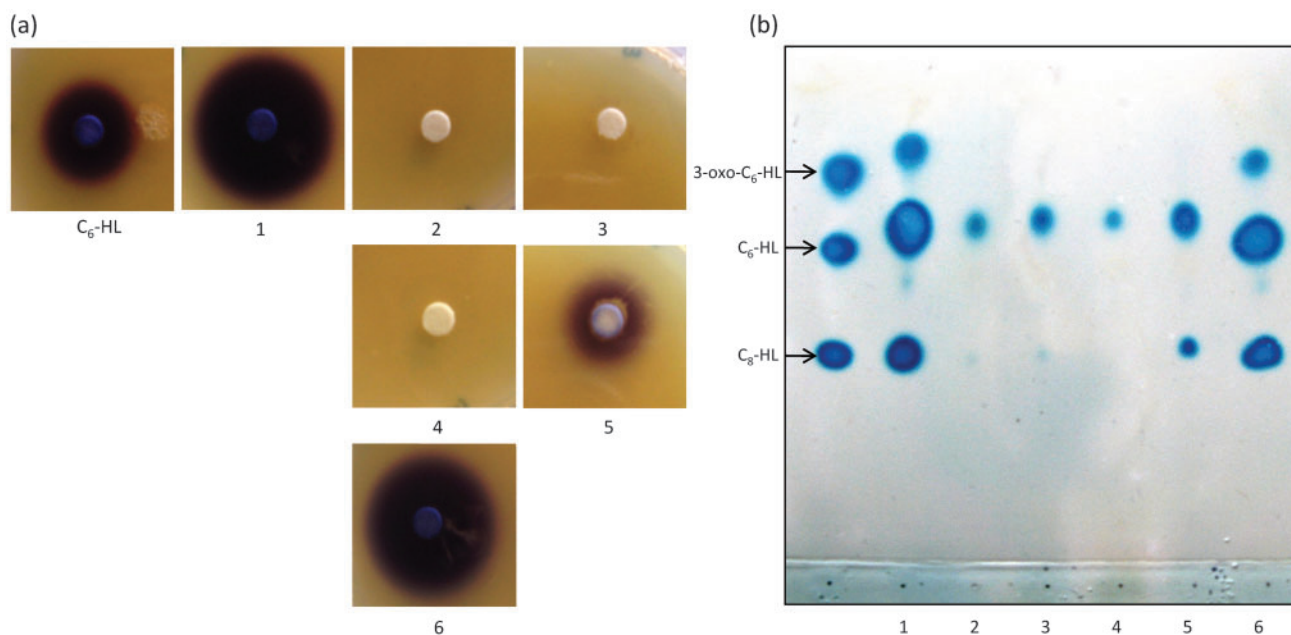


Fig. 1. AHL production in *H. anticariensis* FP35^T (1), strain FP35-11I (2), strain FP35-11I harbouring pJN-HanR (3), strain FP35hanI (4), strain FP35hanI harbouring pJN-HanI (5) and strain FP35hanR (6). AHLs extracted from cultures were visualized on an agar plate (a) by means of the *C. violaceum* CV026 biosensor, and by TLC (b) overlaid with the *A. tumefaciens* NTL4 (pZLR4) indicator strain. Synthetic AHL compounds were used as references: 3-oxo-C₆-HL (4.7 pmol); C₆-HL (80.4 pmol for CV026 and 804 pmol for NTL4); C₈-HL (31.6 pmol).

start of ORF1. A Southern blot analysis confirmed that a single copy of the transposable element had been integrated into the chromosome of this mutant (data not shown).

The results of a homology search for the nucleotide sequence of the two ORFs in the NCBI database showed 26 to 31 % similarity to the *luxR* and *luxI* family genes. ORF1, called *hanR* (for *H. anticariensis luxR*), contained 759 bp and encoded a putative protein of 252 aa with a predicted molecular mass of 28.9 kDa. HanR shared about 31 % identity with LuxR from *Burkholderia glumae* (Devescovi *et al.*, 2007) and 30 % identity with PhzR from *Pseudomonas chlororaphis* strain 30-84 (Khan *et al.*, 2007). Genetic and biochemical studies have identified two conserved domains in LuxR-type proteins: the autoinducer-binding N-terminal domain and the DNA-binding C-terminal domain (Whitehead *et al.*, 2001). Analysis of the putative protein HanR in *H. anticariensis* FP35^T with the SMART program revealed that it contained both domains: an autoinducer-binding domain (amino acids 30–178) and a DNA-binding domain with a helix–turn–helix motif, which is characteristic of the LuxR family (amino acids 189–246) (Letunic *et al.*, 2006) (Supplementary Fig. S1). The second ORF, named *hanI* (for *H. anticariensis luxI*), was located downstream of *hanR* and was transcribed in the same direction (Fig. 2a). The *hanI* gene (645 bp) encoded a 24.4 kDa protein homologous to members of the LuxI family of proteins, which are responsible for AHL synthesis. HanI shared 27 % identity with ExpI from *Erwinia carotovora* (Pirhonen *et al.*, 1993)

and 26 % identity with EanI from *Pantoea ananatis* (Morohoshi *et al.*, 2007). The HanI protein has similar conserved amino acids at the N terminus to those found in other related AHL synthases (Supplementary Fig. S1).

The quorum-sensing *hanR* and *hanI* genes identified are organized in tandem. A similar genetic arrangement has recently been reported in other micro-organisms, such as *R. palustris* (Schaefer *et al.*, 2008), *Gluconacetobacter intermedius* (Iida *et al.*, 2008), *Pseudomonas corrugata* (Licciardello *et al.*, 2007), *Mesorhizobium tianshanense* (Zheng *et al.*, 2006) and *Burkholderia cenocepacia* (Malott *et al.*, 2005). Nevertheless, in other bacteria, such as *Aliivibrio fischeri* (Urbanczyk *et al.*, 2007) (formerly *Vibrio fischeri*), the *luxI* and *luxR* genes are transcribed divergently (Engebrecht & Silverman, 1984).

The *hanRI* genes are co-transcribed

Given that the *hanR* and the *hanI* genes are aligned in the same direction and only 76 bp of intergenic region separates them (Fig. 2a), they may well constitute an operon. To ascertain whether these two genes were part of the same transcriptional unit we carried out RT-PCR analysis using primers based on their intergenic region sequence. Fig. 2(b) shows the predicted fragment corresponding to the intergenic region.

Two possible σ^{70} promoters were identified upstream of the *hanR* and *hanI* regions (Fig. 2c) using the promoter

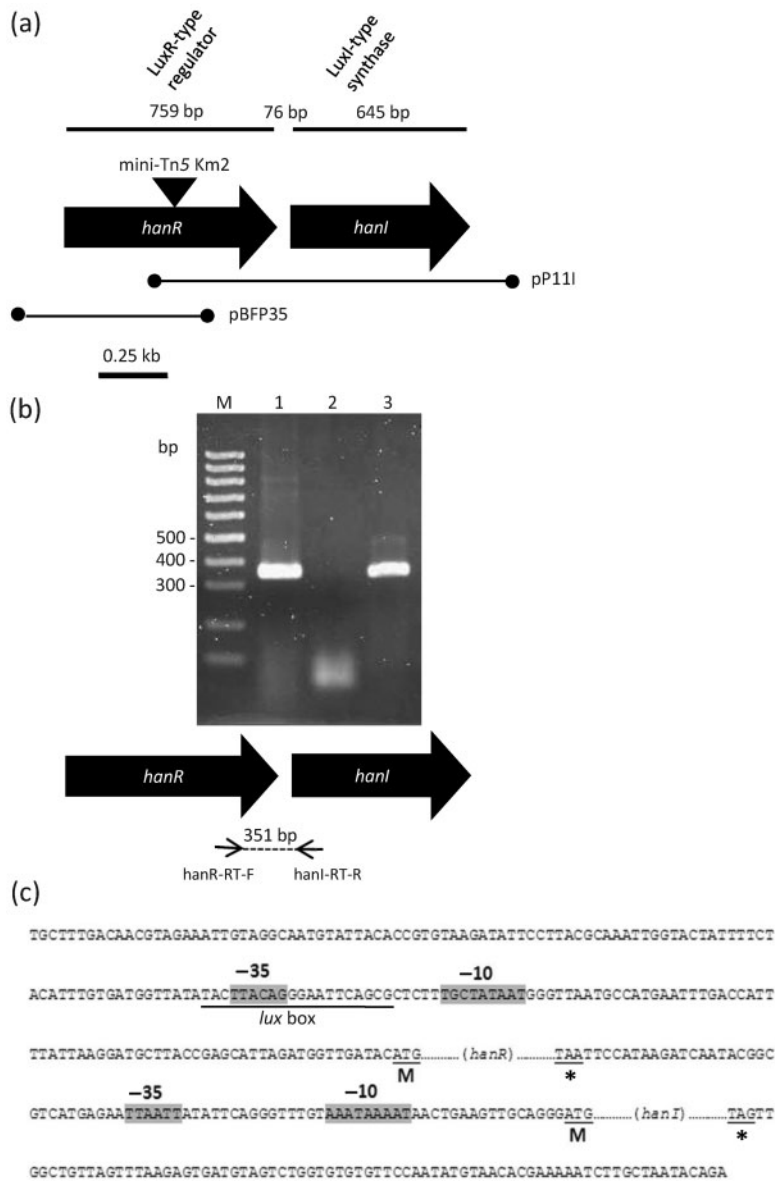


Fig. 2. Transcriptional organization of the *hanR/hanI* locus of *H. anticariensis* FP35^T. (a) Schematic drawing of the *hanR* and *hanI* genetic arrangements in *H. anticariensis* FP35^T. Arrows indicate the proposed direction of transcription. Plasmids used for DNA sequencing, pP111 and pBFP35, containing *Pst*I and *Bam*HI restriction fragments, are indicated on the physical map. The mini-Tn5 Km2 transposon insertion site is indicated by a black triangle. (b) Transcriptional analysis of the *hanR*–*hanI* intergenic region by RT-PCR. Lanes: M, molecular mass marker (100 bp–1 kb); 1, amplification of DNA sample (positive control); 2, amplification of RNA sample without reverse transcriptase (negative control); 3, amplification of RNA sample using reverse transcriptase (expected size of the product, 351 bp). The location of the primers and span of the intergenic region used in the RT-PCR experiment are shown. (c) Sequence of the DNA region upstream of *hanR* and *hanI*, showing the start codon (M) and stop codon (asterisk). A putative *lux* box-like sequence is present in the region upstream of the *hanR* gene.

prediction software BPROM. We then constructed fusions of the putative promoter regions of each gene to a '*lacZ*' gene in the low-copy-number pMP220 vector and transferred it into *H. anticariensis* (see Methods and Table 1). In both cases, promoter activity was detected and measured by β -galactosidase assays: the FP35(pMP-phanR) strain gave a value of 451.49 ± 21.61 Miller units, whereas the FP35 (pMP-phanI) strain gave a value of 72.40 ± 8.21 Miller units [the FP35(pMP220) strain gave a value of 6.94 ± 0.98 Miller units]. Thus our results suggest that the *hanR* and *hanI* genes constitute the same transcriptional unit, although there are promoter activities upstream of each gene.

A putative *lux* box-like sequence, a 20 bp inverted repeat sequence, was found between positions -78 and -98 upstream of the *hanR* start translational site (Fig. 2c). This sequence is homologous to other known *lux*

box sequences (Supplementary Table S2). The *lux* box sequences are thought to represent binding sites of LuxR homologues (Gray *et al.*, 1994) and are located within the promoter regions of many genes regulated by the quorum-sensing system.

The expression of *hanRI* genes is growth phase-dependent

In a previous work we demonstrated that the synthesis of AHLs in *H. anticariensis* FP35^T is growth phase-dependent and that maximum production is reached during the late exponential to stationary phases (Llamas *et al.*, 2005). The relative expression of the *hanR* and *hanI* genes, which was measured by using RT-qPCR, increased 83.6- and 45.1-fold, respectively, by the end of the exponential growth phase. This finding was to be expected and it confirms that

the expression of the *hanR/hanI* system is cell density-dependent, as it is in most quorum-sensing systems (González & Marketon, 2003).

***hanI* gene function**

AHL production by the FP35*hanI* mutant was revealed on an agar plate (Fig. 1a, panel 4) and its pattern was analysed by TLC (Fig. 1b, lane 4), which indicated that it did not produce any detectable levels of AHLs. The slight induction of the NTL4 (pZLR4) indicator strain corresponding to this strain, as shown by TLC, is the same as the signal obtained from a sample prepared from 5 ml uninoculated MY broth medium (Llamas *et al.*, 2005). Therefore, according to these results, HanI would seem to be the sole autoinducer synthase responsible for the production of AHLs in *H. anticariensis* FP35^T. The fact that *H. anticariensis* produces at least four different AHLs is not surprising, as the same has been reported in other bacteria such as *M. tianshanense* (Zheng *et al.*, 2006). It has been suggested that the variability in the acyl chain length of AHLs is not only a function of acyl chain specificity but may also be influenced by the cellular pool of acyl ACPs available (Watson *et al.*, 2002). Moreover, AHL production was almost completely restored in the FP35*hanI* mutant when it was complemented with the *hanI* gene cloned into the pJN105 expression vector (Fig. 1a, panel 5 and b, lane 5). Although the pJN105 plasmid is a broad-host-range expression vector, it has not been used before in studies with moderate halophiles and so it may be that AHLs were not fully produced because the vector was not properly expressed.

The transcription of *hanI* synthase from the *hanI* promoter was measured in the FP35*hanI* mutant by β -galactosidase assays, which indicated that it was unaffected by adding exogenous AHLs to the culture (Supplementary Fig. S2). Since we were unable to detect autoinduction in the synthesis of AHLs in *H. anticariensis* FP35^T, we suggest that the classic *A. fischeri* autoinduction of the *hanI* gene does not take place in this strain in the same way as has been described in other bacteria (Andersson *et al.*, 2000; Christensen *et al.*, 2003; Nasser *et al.*, 1998; Throup *et al.*, 1995). Moreover, No *lux* box-like regulatory element could be identified within the region upstream of the translational start codon of the *hanI* gene.

***hanR* gene function**

The FP35*hanR* mutant produced quantities of signal molecules similar to those of the wild-type (Fig. 1a, panel 6 and b, lane 6). This result was unexpected, since the majority of *luxR* mutants described in other bacteria produce fewer or no AHLs. Since the FP35*hanR* mutant was completely defective in *hanR* expression (Supplementary Fig. S3a) and the level of expression of the *hanI* gene was similar to that of the wild-type strain (Supplementary Fig. S3a), it would seem that the HanR protein is not essential for AHL production in *H. anticariensis* FP35^T.

In contrast, the FP35*hanR* mutant synthesized higher quantities of AHLs than FP35-11I (mini-Tn5:: *hanR*) (Fig. 1b, lanes 2 and 6), another mutant in which the *hanR* gene is not transcribed (Supplementary Fig. S3a). This result may well be due to a polar effect caused by the transposon on the *hanI* gene downstream, since the *hanI* gene expression level is lower than that of FP35^T or FP35*hanR* (Supplementary Fig. S3a). In fact, AHL production was not restored in the FP35-11I mutant when it was complemented with the pJN-HanR plasmid (Fig. 1a, panel 3 and b, lane 3).

The presence of a putative *lux* box-like sequence upstream of the *hanR* start codon suggested that the expression of *hanR* was AHL-dependent. Nevertheless, we found that transcription of the *hanR* gene was induced in the AHL-deficient strain FP35*hanI* to a degree similar to that of the wild-type strain even when crude extracts of AHLs were added to the bacterial culture (Supplementary Fig. S3b). This result suggests that *hanR* is expressed from its own promoter but does not seem to be modulated by the AHL signalling molecules under our assay conditions. One explanation is that HanR might have the capacity to act as a regulator of its own expression even without binding to the AHLs. Another explanation is that there might be one or more additional transcriptional LuxR regulators in *H. anticariensis* FP35^T. These unpaired LuxR family proteins have been well characterized and shown to be integrated with the resident complete AHL quorum-sensing regulatory network (Patankar & González, 2009; Subramoni & Venturi, 2009). This has been reported in the SinRI quorum-sensing system of *Sinorhizobium meliloti*, where the ExpR regulator, an unpaired LuxR homologue, binds to a site in the *sinR*–*sinI* intergenic region, which affects the transcription of the *sinI* synthase gene (Hoang *et al.*, 2004; McIntosh *et al.*, 2009). More studies must be undertaken in *H. anticariensis* FP35^T to test these hypotheses.

Having identified and characterized the quorum-sensing *hanR/hanI* system involved in the synthesis of AHLs in *H. anticariensis* FP35^T, we are now turning our attention to the role of this type of cell density-dependent gene regulation in the extreme environments in which *Halomonas* species live and in which they are ubiquitous. Within this context, *Halomonas* species are in general considered to be free-living bacteria, and many of them produce exopolysaccharides that allow them to form biofilms; cell-to-cell communication may then be necessary to regulate the bacterial community in any given niche. To our knowledge this is the first report describing genetically the quorum-sensing system in a moderately halophilic bacterium, and it confirms previous evidence for cell-to-cell communication amongst micro-organisms in an extreme environment (Llamas *et al.*, 2005).

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