Genetic and phenotypic analysis of the GacS/GacA system in the moderate halophile *Halomonas anticariensis*

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A multisensory, hybrid histidine kinase (HK) and a response regulator (RR), which together may well constitute a two-component regulatory system (TCS), have been located in *Halomonas anticariensis* FP35¹ by transposon mutagenesis. This TCS is homologous to the GacS/GacA system described for many Gram-negative bacteria. An analysis of crude N-acylhomoserine lactone (AHL) extracts from cultures of FP35gacS and FP35gacA mutants showed that they produced lower quantities of AHLs than the wild-type strain. In addition, RT-PCR analysis revealed a considerable decrease in the expression of the quorum-sensing (QS) genes *hanR* and *hanI* compared with the wild-type strain. This result indicates that the GacS/GacA TCS exerts a positive effect upon the QS HanR/HanI system and suggests its integral involvement in the intercellular communication strategies of this bacterium. We have also demonstrated the influence of GacS and GacA upon exopolysaccharide production and biofilm formation, in which this regulatory machinery appears to play a key role in an overall system that co-ordinates gene expression and behaviour in *H. anticariensis* FP35¹ in response to environmental conditions.

INTRODUCTION

The overall complexity of gene expression has been the subject of extensive investigation in many micro-organisms. Two-component systems (TCSs) are used by bacteria to perceive different environmental stimuli, transmit information amongst cells and convert this information into suitable modifications in gene expression in response to changing environments (Gao et al., 2007; Jung et al., 2012). A typical TCS consists of a transmembrane-sensor histidine kinase (HK), responsible for transferring a phosphoryl group to the receiver domain of the cognate response regulator (RR). Among TCSs the GacS/GacA system is well preserved in a variety of Gram-negative bacterial genera, such as *Acinetobacter*, *Azotobacter*, *Erwinia*, *Escherichia*, *Legionella*, *Pseudomonas*, *Salmonella*, and *Serratia* and *Vibrio*. The GacS/GacA TCS has been reported to regulate an array of phenotypes such as the production of virulence factors, plant-growth promotion, swarming motility, biofilm formation, exopolysaccharide (EPS) biosynthesis, production of secondary metabolites and quorum sensing (QS) signal molecules and the secretion of proteins and enzymes (Gooderham & Hancock, 2009; Heeb & Haas, 2001).

The GacS/GacA TCS interacts within a complex regulatory network and operates via different pathways in Gram-negative bacteria (Galperin, 2006). One of these is the alternative sigma factor σ^38 (RpoS), which plays a key role in the survival of bacteria during starvation or exposure to stress conditions and is required for the expression of many genes in the stationary growth phase (virulence factors and secondary metabolites). It may not, however, be essential for cell viability (Hengge-Aronis, 2002; Schuster et al., 2004; Venturi, 2003). A second pathway involves the intervention of the GacS/GacA TCS in the QS cascade. The QS system is a population-density-dependent gene-expression mechanism that involves the production of signal molecules known as autoinducers (reviewed by Gonzalez & Marketon, 2003; Ng & Bassler, 2009; Parker & Sperandio, 2009). The autoinducer signals from Gram-negative *Proteobacteria* are generally N-acylhomoserine lactones (AHLs), which are commonly produced by an
autoinducer synthase belonging to the LuxI protein family. The QS system involves the accumulation of AHL molecules in the extracellular medium until a critical value is reached, at which point the AHLs bind to a transcriptional activator, which triggers the expression of target genes, including the luxI gene, leading in turn to the production of more AHLs (Eberhard et al., 1991; Fuqua et al., 1994; Swift et al., 1999). It has been suggested that the three systems, GacS/GacA, QS and RpoS, interact in a complex regulatory network, which is probably modulated according to environmental conditions. In some Pseudomonas species the QS and RpoS systems regulate each other whilst GacA positively regulates the production of AHLs (Bertani & Venturi, 2004). A third pathway begins with the GacS/GacA TCS triggering the transcription of one or several genes that encode small untranslated RNAs (sRNAs) (Bejerano-Sagie & Xavier, 2007; Lapouge et al., 2008). One example of this mechanism has been reported in Vibrio cholerae, where the VarS/VarA system, which is homologous to GacS/GacA, controls the expression of three sRNAs, known as CsrB, CsrC and CsrD, when the cultures reach high cell density. These three small RNAs act together and antagonize the function of CsrA, a post-transcriptional regulator that stimulates the translation of its gene targets by binding mRNAs (Lenz et al., 2005).

The bacteria most frequently isolated from hypersaline environments tend to be members of the Gram-negative, halophilic genus Halomonas (Quesada et al., 2004). Halomonas anticariensis FP35\(^T\) was first identified in saline soils at Fuente de Piedra, Málaga, in southern Spain. This bacterium characteristically grows over a wide salt range of 0.5% to 15% w/v and excretes significant quantities of EPS when the early stationary growth phase is reached (Mata et al., 2006). We have reported that this bacterium produces a series of AHLs of many sizes [C\(_4\)-homoserine lactone (HSL), C\(_6\)-HSL, C\(_8\)-HSL and C\(_{12}\)-HSL] (Llamas et al., 2005) and have recently identified and characterized the QS genes hanR/hanI involved in their production (Tahrioui et al., 2011). Since we were unable to detect autoinduction of the synthesis of the AHLs in our bacterium we speculated that its QS system may well be integrated in a more complex, overall intercellular communication system, in which, among other functions, the GacS/GacA TCS controls the production of signal molecules.

In this work we have identified and characterized a possible multi-hybrid, sensor histidine kinase GacS and a response-regulator GacA, which together may well constitute a TCS in H. anticariensis FP35\(^T\). The GacS/GacA TCS seems to be an all-embracing regulatory system that exerts a positive effect upon the production of AHLs, EPS synthesis and the formation of biofilm.

**METHODS**

**Bacterial strains, media and plasmids.** 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 MH medium (Moraine & Rogovin, 1966) modified with a balanced mixture of sea salts (Rodriguez-Valera et al., 1981). Escherichia coli strains were grown at 37 °C in LB broth (Sambrook & Russel, 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\(^{-1}\). The pH of the media was adjusted to 7.2 with NaOH (1 M). Solid media contained an additional 20 g Bacto agar (Difco) I \(^{-1}\). Agrobacterium tumefaciens NTL4 (pZLR4) was cultured at 30 °C in Luria–Bertani broth supplemented with 2.5 mM CaCl\(_2\) and 2.5 mM MgSO\(_4\) (LB/MC) and in MGM minimal medium (Llamas et al., 2005) containing 50 μg gentamicin ml\(^{-1}\). Chromobacterium violaceum CV026 was grown at 30 °C in LB medium.

**Transposon mutagenesis and cloning of the gacS/gaca ORFs.** Transposon mutagenesis was carried out via conjugation by biparental mating, as described in previous publications (Llamas et al., 2000; Tahrioui et al., 2011). To isolate the DNA fragments bearing the mini-Tn5Km2 insertion, which encode the Km resistance gene, genomic DNA samples from two AHL-deficient mutants, H. anticariensis FP35-48XII and FP35-79X, were 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 & Russel, 2001). Fragments containing the mini-Tn5Km2 insertion were analysed by Southern hybridization using a digoxigenin DNA labelling and detection kit (Boehringer) as described in previous publications (Llamas et al., 1997, 2003). Chromosomal DNA fragments from H. anticariensis FP35-48XII digested with SalI were selected and ligated into pGEM-T to create p848XII. The genome sequence located downstream of the known nucleotide sequence was obtained by inverse PCR (Ochman et al., 1988; Llamas et al., 2003). Two plasmids, pFP35 and pEFP35, were isolated. Chromosomal DNA fragments from H. anticariensis FP35-79X digested with EcoRI and SalI were chosen and ligated into pBlueScript SK (+) and pGEM-T to create pE79X and pS79X respectively. 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 DNA sequences thus obtained were analysed using a BLAST search of the GenBank database. Promoter regions were predicted with the BPROP software [Softberry (http://linux1.softberry.com/berry, phtml)]. The conserved domains of the predicted proteins GacS and GacA were identified using the SMART program (Letunic et al., 2006). A phylogenetic tree of GacS- and GacA-like proteins was constructed using version 4 of the MEGA software (Tamura et al., 2007) after multiple alignments of the data by CLUSTAL W (Thompson et al., 1997). Distances and clustering were determined according to the neighbour-joining method and bootstrap values were measured on the basis of 1000 replications. Sequences of GacS/Gaca-like proteins found experimentally in Gammaproteobacteria were retrieved from GenBank.

**DNA manipulation.** All molecular techniques were conducted using standard protocols (Sambrook & Russel, 2001). The primers used in this study are listed in Table S1 available with the online version of this paper.

**Construction of the uvrC, gacS and gacA mutants.** The uvrC and gacS chromosomal genes were disrupted in a single step by Campbell-type integration of the entire plasmid by homologous recombination using the suicide plasmid pVIK112 (Kalogeraki & Winans, 1997). Briefly, 343 bp and 458 bp internal segments of the uvrC and gacS genes respectively were amplified from H. anticariensis FP35\(^T\) chromosomal DNA using the primers uvrC-EcoRI-F and uvrC-XbaI-R for uvrC and gacS-Sub-EcoRI-F and gacS-Sub-XbaI-R for gacS.
Table 1. Bacterial strains and plasmids used

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>H. anticariensis strains</strong></td>
<td></td>
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<tr>
<td>FP35 (^1) (= CECT 5854 (^T))</td>
<td>Wild-type isolate, mucoid</td>
<td>Martinez-Canovas et al. (2004a)</td>
</tr>
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<td>FP35-R</td>
<td>Spontaneous rifampicin-resistant mutant of FP35, Rif(^r)</td>
<td>Tahrioui et al. (2011)</td>
</tr>
<tr>
<td>FP35-48XII</td>
<td>FP35-R containing gacS::mini-Tn5Km2, Rif(^r), Km(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>FP35-79X</td>
<td>FP35-R containing uvrC::mini-Tn5Km2, Rif(^r), Km(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>FP35gacS</td>
<td>FP35-R containing gacS::lacZ, Rif(^r), Km(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>FP35gacA</td>
<td>FP35-R containing gacA::Km, Rif(^r), Km(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>FP35uvrC</td>
<td>FP35-R containing uvrC::lacZ, Rif(^r), Km(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>FP35GacS (pJN-GacS)</td>
<td>FP35-R containing gacS::lacZ and pJN-GacS, Rif(^r), Km(^r), Gm(^r)</td>
<td>This work</td>
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<td>FP35GacA (pJN-GacA)</td>
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<td>This work</td>
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<tr>
<td>FP35-79X (pJN-GacA)</td>
<td>FP35-R containing uvrC::mini-Tn5Km2 and pJN-GacA, Rif(^r), Km(^r), Gm(^r)</td>
<td>This work</td>
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<tr>
<td><strong>A. tumefaciens strain</strong></td>
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<td>NTL4 (pZLR4)</td>
<td>NT1 derivative bearing a traG::lacZ reporter fusion, Gm(^r)</td>
<td>Shaw et al. (1997)</td>
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<td><strong>C. violaceum strain</strong></td>
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<tr>
<td>CV026</td>
<td>CV017 derivative containing cviI::Tu5xyLe, Km(^r)</td>
<td>McClean et al. (1997)</td>
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<td><strong>E. coli strains</strong></td>
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<tr>
<td>DH5(\alpha)</td>
<td>F(^{-}) #80d lacZ(\Delta)M15 (lacZY-argF(U169) deoR recA1 endA1 hsdR17((K_R) m(K_R)) supE44 λ(^{-}) thi-1 girA96 relA1)</td>
<td>Laboratory collection</td>
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<td>S17-1 (\lambda)pir</td>
<td>RK2 (\mu) regulon, pir, host for (\mu)-dependent plasmids</td>
<td>Miller &amp; Mekalanos (1988)</td>
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<td>XL1-Blue</td>
<td>supE44, hsdR17, recA1 gyrA46 thi-1 relA1 lac(^{-})F(^{\prime}) [proAB + lacIQ lacZAM15 Tn10 ((T_C))]</td>
<td>Bullock et al. (1987)</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUT mini-Tn5Km2</td>
<td>Ap(^r), Km(^r), ori R6K, oriT RP4</td>
<td>de Lorenzo et al. (1990)</td>
</tr>
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<td>pGEM-T</td>
<td>High-copy-number cloning vector, Ap(^r), bla, lacZ</td>
<td>Promega</td>
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<td>pBluescript SK(+)</td>
<td>Ap(^r), lacZ, pMB89 replicon</td>
<td>Stratagene</td>
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<tr>
<td>pUC19</td>
<td>Ap(^r), ColE1 replicon</td>
<td>Sambrook &amp; Russel (2001)</td>
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<td>pS48XII</td>
<td>pUC19 with a 4.4 kb SauI fragment bearing mini-Tn5Km2 insertion, Ap(^r), Km(^r)</td>
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<td>pFP35</td>
<td>pGEM-T with a 1.3 kb inverse PCR product amplified from a 2.2 kb fragment from strain FP35 genome, Ap(^r)</td>
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<td>pEFP35</td>
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<td>pE79X</td>
<td>pBluescript SK(+) with 4 kb EcoRI fragment bearing mini-Tn5Km2 insertion, Ap(^r), Km(^r)</td>
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<tr>
<td>pS79X</td>
<td>pGEM-T with 2.5 kb SauI fragment bearing mini-Tn5Km2 insertion, Ap(^r), Km(^r)</td>
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<td>pVIK112</td>
<td>lacZ(\Delta) for disruption and transcriptional fusion, Km(^r)</td>
<td>Kalogeraki &amp; Winans (1997)</td>
</tr>
<tr>
<td>pVIKgacS</td>
<td>pVIK112 containing an internal gacS fragment, transcriptional fusion, Km(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>pVIKuvrC</td>
<td>pVIK112 containing an internal uvrC fragment, transcriptional fusion, Km(^r)</td>
<td>This work</td>
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<tr>
<td>pGacA</td>
<td>pGEM-T bearing gacA and surrounding region</td>
<td>This work</td>
</tr>
<tr>
<td>pGacA::Km</td>
<td>pGacA bearing gacA::Km</td>
<td>This work</td>
</tr>
<tr>
<td>pJQ200SK</td>
<td>Shuttle vector bearing sacB</td>
<td>Quandt &amp; Hynes (1993)</td>
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<tr>
<td>pGacA::Km/sacB</td>
<td>pJQ200SK bearing gacA::Km</td>
<td>This work</td>
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<td>pJN105</td>
<td>araC-P_BAD cassette cloned in pBBR1MCS5</td>
<td>Newman &amp; Fuqua (1999)</td>
</tr>
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<td>pJN-GacS</td>
<td>pJN105 containing intact gacS, Gm(^r)</td>
<td>This work</td>
</tr>
<tr>
<td>pJN-GacA</td>
<td>pJN105 containing intact gacA, Gm(^r)</td>
<td>This work</td>
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which contain EcoRI and XbaI restriction sites at their respective 5′ ends (Table S1). PCR entailed 30 cycles of 30 s at 95°C, 30 s at 68°C and 45 s at 72°C. The PCR fragments were purified, digested with EcoRI and XbaI and cloned into the suicide plasmid pVIK112. The resulting plasmids, pVIKuvrC and pVIKgacS, were subsequently transformed separately into E. coli S17-1 \(\lambda\)pir and transferred into a rifampicin-resistant FP35 derivative (FP35-R) by biparental mating, where the lacZ gene disrupted the uvrC and gacS genes by single
recombination. The *H. anticariensis* transconjugants were selected by plating them on SWYE medium containing 2% (w/v) salts, rifampicin (50 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) (Kalogeraki & Winans, 1997; Llamas et al., 2004; Tahiroui et al., 2011) (Table 1). These mutants were designated FP35gacS and FP35uvrC.

To disrupt *gacA* we firstly amplified from the chromosomal DNA of FP35 a 1.7 kb fragment containing the *gacA* ORF using the primers GacA-BamHI-F and GacA-BamHI-R (Table S1), both containing a *BamHI* restriction site at their 5’ ends, and then cloned the purified PCR product into pGEM-T to create pGacA. PCR entailed 30 cycles of 30 s at 95°C, 30 s at 65°C and 2 min at 72°C. The kanamycin cassette was amplified using the primers Km-Hpal-F and Km-Hpal-R, both containing a *Hpal* restriction site at their 5’ ends, from pVK112 (Kalogeraki & Winans, 1997). PCR entailed 30 cycles of 30 s at 95°C, 30 s at 60°C and 1 min at 72°C. The *Hpal*-kanamycin cassette was then ligated into the only *Hpal* site of the *gacA* ORF to create pGacA:: Km. The *gacA:: Km* disruption was then transferred from pGacA:: Km as a *BamHI* fragment into pJQ200SK, creating pGacA:: Km/sacB, which was then transformed into S17-1 *Δpir* and transferred into the rifampicin-resistant *H. anticariensis* FP35R by biparental mating. *H. anticariensis* clones bearing the *gacA* disruption (obtained by replacement) were selected by plating them on SWYE medium containing 2% (w/v) sea salts, rifampicin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹) and 5% (w/v) sucrose. This mutant was designated FP35gacA.

**Cloning of the gacS and gacA ORFs in a multicopy plasmid.** The ORFs and the predicted promoter regions of *gacS* and *gacA* were amplified from *H. anticariensis* FP35 chromosomal DNA by using the primers GacS-C-BamHI-F and GacS-C-BamHI-R, and GacA-C-BamHI-F and GacA-C-BamHI-R respectively, which contain *BamHI* and *XbaI* restriction sites at their respective 5’ ends (Table S1). The PCR fragments were purified, digested with *BamHI* and *XbaI* and cloned into the broad-host-range expression vector pJN105 (Newman & Fuqua, 1999) to create pJN-GacS and pJN-GacA. The pJN-GacS and pJN-GacA plasmids were transformed separately into *E. coli* S17-1 *Δpir* and then the pJN-GacS plasmid was transferred by biparental mating into the mutant strain FP35gacS and the pJN-GacA plasmid into the mutants FP35-79X and FP35gacA respectively to be used in complementation analysis. All constructs were confirmed by PCR.

**RNA extraction and RT-PCR assays.** Total RNA from bacterial cultures growing at the stationary phase (OD₅₆₀ 1.4) was extracted 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 µg total RNA at a final volume of 50 µl using the SuperScript III One-Step RT-PCR System with Platinum *Taq* according to the manufacturer’s instructions (Invitrogen). cDNA was synthesized at 55°C for 30 min. Denaturation took place over 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 (Table S1). Positive and negative controls were included in all the assays. The 16S rRNA gene was used as a control for normalization. RT-PCR products were visualized by gel electrophoresis.

**Extraction and detection 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 strains *A. tumefaciens* NTL4 and *C. violaceum* CV026 was diluted 1:100 in 5 ml LB/MC and LB fresh media and poured onto AB medium containing 80 µg X-Gal ml⁻¹ and LB agar respectively. Once the plates were dry, paper disks 5 mm in diameter were placed on an agar plate and the AHL samples applied. The assay plates were incubated overnight at 32°C and the appearance of pigment around the filter was determined.

**N-Acylhomoserine lactone β-galactosidase assays.** An overnight culture of the indicator strain *A. tumefaciens* NTL4 (pZLR4) was grown in LB/MC broth containing gentamicin and sub-cultured (1:100) in MGM minimal medium. A crude extract of AHLs from *H. anticariensis* FP35, FP35uvrC, FP35gacS or FP35gacA was then added to the medium and grown at 30°C until reaching OD₅₆₀ = 0.5. β-Galactosidase activity was measured by Miller’s method (Miller, 1972) using ONPG as substrate. Each sample was assayed in triplicate and each experiment was repeated at least three times.

**EPS production.** The bacterial strains were cultured for 5 days at a stirring rate of 100 r.p.m. at 32°C in MY complex medium supplemented with 7.5% (w/v) sea salts. The EPSs were isolated using the method described in previous publications (Quesada et al., 1993, 1994). Briefly, the culture was centrifuged and the supernatant precipitated with cold ethanol before being ultracentrifuged, dialysed against distilled water and lyophilized.

**Electron microscopy.** Ultrathin sections of bacterial cells were negatively stained as described elsewhere (Bouchotroch et al., 2001).

**Quantitative biofilm assay.** To assess the propensity of bacterial strains to produce biofilms we carried out crystal-violet-adhesion assays as described by O’Toole & Kolter (1998). Briefly, overnight cultures were inoculated at a ratio of 1:100 into a fresh medium and grown for 24 and 48 h in a 96-well polystyrene microtitre plate. Cell growth was determined from OD₅₆₀. Biofilm was measured by discarding the medium, rinsing the wells with water and staining any bound cells with crystal violet. The dye was dissolved in 95% (v/v) ethanol and A₅₉₀ was determined using a microtitre-plate reader (Tecan). In each experiment background staining was corrected by subtracting the crystal violet bound to uninoculated controls. Each assay was conducted at least in triplicate.

**Nucleotide sequence accession number.** The *gacS* and *gacA* DNA sequences reported here have been deposited in the GenBank database under accession numbers JQ437497 and EF070731 respectively.

**RESULTS**

**Isolation and characterization of the AHL-deficient strains FP35-48XII and FP35-79X reveals GacS/GacA homologues in *H. anticariensis* FP35**

Two strains of *H. anticariensis* FP35 partially deficient in AHL, FP35-48XII and FP35-79X, were chosen from 2000 transconjugants after streaking them against the indicator strains *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* NTL4 (pZLR4). The mini-Tn5Km2 genonomic mutants showed less induction of both indicator strains compared with the wild-type (Fig. 1). A Southern-blot analysis confirmed that a single copy of the
transposable element had been integrated into the chromosome of each of these mutants (data not shown).

To determine which genes had been affected by insertion of the mini-Tn5Km2 into mutant FP35-48XII, the nucleotide sequences of the chromosomal DNA in the three recombinant plasmids, pS48XII, pFP35 and pEFPP35, were analysed and the four complete ORFs found (Fig. 2a) were submitted to a homology search in the National Center for Biotechnology Information (NCBI) database. The transposon insertion site within the FP35-48XII genome was located after base pair 1585 from the translation start of ORF2. ORF1 is predicted to encode a 31.9 kDa protein involved in cysteine synthesis. ORF2 (2745 bp), transcribed in the opposite direction to the other three ORFs, is predicted to encode a 100.1 kDa protein. This ORF shared 54 % identity with a possible TCS multi-sensor, hybrid, histidine kinase protein from Chromohalobacter salexigens, 53 % identity with one from Halomonas elongata and 37–40 % identity with the hybrid, histidine kinase proteins from members of the genus Pseudomonas. We designated this ORF gacS in the same way as its homologues. Analysis of the predicted GacS protein in H. anticariensis FP35T with the SMART program revealed that it contained two transmembrane domains (amino acids 5–27 and 154–176), a linker histidine kinase, adenyl cyclase, methyl-binding protein, phosphatase (HAMP) domain (amino acids 177–229), a (HisKA) His kinase A (phosphoacceptor) domain (amino acids 269–334), a histidine kinase-like ATPases domain (amino acids 381–497), a cheY-homologous receiver (REC) domain (amino acids 642–756) and a histidine phosphotransfer (HPT) domain (amino acids 797–891). ORFs 3 and 4 shared 67 % to 56 % and 79 % to 76 % identity with 4’-phosphopantetheinyltransferase and pyridoxine 5’-phosphate synthase genes from Halomonas species respectively.

With regard to the mutant FP35-79X, the DNA sequence of two contiguous EcoRI and SalI fragments (4 kb and 2.5 kb, subcloned as pE79X and pS79X respectively, as described in Methods) showed the presence of five ORFs, two of which (1 and 5) were partial sequences (Fig. 2b). The transposon insertion site within the H. anticariensis strain FP35-79X genome was located after codon 19 of ORF5. This partial ORF5, of 537 bp, shared 83 % to 59 % identity with sequences of the excinuclease ABC subunit C, which participates in DNA repair after UV damage and is also found in genera such as Halomonas, Chromohalobacter, Pseudomonas and Marinobacter. Upstream of the uvrC homologue (ORF5), in the same direction was ORF4 (654 bp), which encodes a putative protein of 218 amino acids that shared 80 % identity with the two-component LuxR family transcriptional regulator of C. salexigens; 73 % to 61 % identity to the response regulator GacA of Halomonas species and 60 % to 57 % identity with several known response-regulator GacA homologues, such as GacA of Pseudomonas fluorescens and Pseudomonas chlororaphis subsp. aureofaciens. We have designated this ORF gacA in the same way as its homologues. An analysis of the predicted GacA protein in H. anticariensis FP35T with the SMART program revealed that it contained two transmembrane domains (amino acids 5–27 and 154–176), a linker histidine kinase, adenyl cyclase, methyl-binding protein, phosphatase (HAMP) domain (amino acids 177–229), a (HisKA) His kinase A (phosphoacceptor) domain (amino acids 269–334), a histidine kinase-like ATPases domain (amino acids 381–497), a cheY-homologous receiver (REC) domain (amino acids 642–756) and a histidine phosphotransfer (HPT) domain (amino acids 797–891). ORFs 1, 2 and 3 shared 71 % to 63 %, 64 % to 60 % and 75 % to 69 % identity to the GntR family.

**Fig. 1.** AHL production by *H. anticariensis* FP35T and strains FP35-48XII and FP35-79X. AHLs extracted from cultures were visualized on agar plates by means of the *C. violaceum* CV026 and *A. tumefaciens* NTL4 (pZLR4) indicator strains.
transcriptional regulator, chromate transport A and chromate transport B genes from *Halomonas* species respectively.

We made a phylogenetic analysis of the predicted GacS and GacA proteins in *H. anticariensis* FP35T and the GacS and GacA homologues held in databases concerning other micro-organisms, including pseudomonads and enteric bacteria (Fig. S1). The two trees generated by the neighbour-joining method were similar in topology, with the sequences from halophilic strains grouping together in both cases and being separated from the rest of the *Gammaproteobacteria* analysed.

**The hanR/hanI QS system is positively regulated by the GacS/GacA TCS**

The mutant FP35-79X produced almost no AHLs. This may well be due to a polar effect caused by the transposon inserted within *uvrC* (ORF5) on the *gacA* gene (ORF4), which is located upstream of *uvrC* and forms part of the same transcriptional unit (Fig. S2). To confirm this hypothesis the mutant FP35-79X was complemented with the *gacA* gene cloned into the pJN105 expression vector (see Methods) and its AHL production was determined by a β-galactosidase assay using the indicator strain *A. tumefaciens* NTL4 (pZLR4). As can be seen in Fig. 3, AHL production was completely restored in strain FP35-79X (pJN-GacA). We also constructed a mutation of the *uvrC* gene and determined AHL production in the FP35-*uvrC* mutant in the same way. The FP35-*uvrC* mutant produced higher quantities of AHLs than those synthesized by the FP35-79X mutant but less than the wild-type (Fig. 3).

To study the role of the TCS in the regulation of QS gene expression we constructed *gacS* and *gacA* mutants of *H. anticariensis* FP35T (FP35*gacS* and FP35*gacA*). We first compared the accumulation of AHL signal molecules by β-galactosidase assays by measuring the induction of the lacZ reporter system. The AHL in each case was obtained by preparing crude extracts from a stationary-phase culture. Our results indicated that the FP35-*gacS* and FP35-*gacA* mutants produced significantly smaller quantities of AHL signal molecules than the wild-type strain (Fig. 4a). We then went on to quantify by RT-PCR the expression of the autoinducer synthase *hanI* and the transcriptional regulator *hanR* in *H. anticariensis* FP35T and its derivative mutants. RT-PCR analysis revealed that the expression of *hanI* and *hanR* genes in FP35*gacS* and FP35*gacA* was drastically reduced compared with the wild-type strain (Fig. 4b). These results suggest that the GacS/GacA TCS positively regulates the *hanR/hanI* QS system and consequently AHL production.
Effect of GacS/GacA TCS on EPS production

The mutant FP35\textsubscript{gacS} and FP35\textsubscript{gacA} colonies were smooth but not mucoid, in contrast to the mucous colonies produced by the wild-type strain (Fig. 5a). This observation led us to examine the effect of the GacS/GacA TCS on EPS production. To this end we cultured both mutants and the wild-type and extracted their EPSs. Strains FP35\textsuperscript{T}, FP35\textsubscript{gacS} and FP35\textsubscript{gacA} produced 37.50, 21.67 and 16.67 mg EPS 100 ml\textsuperscript{-1} of culture medium (167.5, 95.2 and 78.0 mg EPS g dry cell weight\textsuperscript{-1}) respectively. These results demonstrated that the disruption of the gacS and gacA genes resulted in a significant decrease in EPS production. The mutants produced around 42 % and 55 % less polymer than the wild-type strain. We also studied EPS production under a transmission electron microscope by staining the bacterial cells with ruthenium red. Fig. 5(b) shows that the cells of FP35\textsuperscript{T} and its mutants are similar in size but that there are no EPSs present in the surrounding medium.

Influence of the inactivation of gacS and gacA on biofilm formation in H. anticariensis FP35\textsuperscript{T}

The inability of the mutants FP35\textsubscript{gacS} and FP35\textsubscript{gacA} to produce EPS should have a concomitant effect on their capacity to develop biofilm. To confirm this we determined the adhesion capacity of the wild-type and the mutant strains after 24 and 48 h incubation at 32 °C (Fig. 6). There were no significant differences in bacterial growth rate between the wild-type and mutant strains. Nevertheless, there was a significant reduction in biofilm attached to the wells containing the mutants compared with those containing the wild-type after 48 h growth. These observations suggest that the GacS/GacA TCS regulates biofilm formation in H. anticariensis FP35\textsuperscript{T}.

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**Fig. 3.** AHL production by H. anticariensis FP35\textsuperscript{T} and strains FP35\textsubscript{uvrC}, FP35-79X and FP35-79X (pJN-GacA). AHLS extracted from cultures were quantified by measuring β-galactosidase activity using the AHL indicator strain A. tumefaciens NTL4(pZLR4). The values are means ± sds (n=3).

**Fig. 4.** Effect of the GacS/GacA TCS on AHL production: (a) quantification of AHL levels produced by H. anticariensis FP35\textsuperscript{T} and its derivative mutants FP35\textsubscript{gacS} and FP35\textsubscript{gacA}, as assayed by measuring β-galactosidase activity with the AHL indicator strain A. tumefaciens NTL4(pZLR4) [the values are means ± sds (n=3)]; (b) expression of the hanI and hanR genes was evaluated by RT-PCR in the wild-type H. anticariensis FP35\textsuperscript{T} and in its derivative mutants FP35\textsubscript{gacS} and FP35\textsubscript{gacA}. 

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Restoration of GacS and GacA regulated phenotypes in *H. anticariensis* FP35T

To test our hypothesis that the phenotypic changes in *H. anticariensis* resulted from the inactivation of the *gacS* and *gacA* genes we constructed pJN-GacS and pJN-GacA, which contain the *gacS* and *gacA* ORFs as well as the presumed promoter region of each ORF. These plasmids were transferred into the FP35*gacS* and FP35*gacA* mutants respectively and tested for various phenotypes. The reduction in AHL accumulation was restored in FP35*gacA* (pJN-GacA) to an even higher level than in the wild-type strain, whilst in FP35*gacS* (pJN-GacS) this phenotype was not fully restored (Fig. 7a). EPS production and biofilm development were restored in each of the complemented strains to a level similar to that of the wild-type (Fig. 7b and c).

DISCUSSION

EPS-producing *Halomonas* strains are widespread in salt-terns, saline soils, marshes and seawater (Amjres et al., 2011; Bouchotroch et al., 2001; González-Domenech et al., 2008a, b; Llamas et al., 2011; Martínez-Cañovas et al., 2004a, b; Martínez-Checa et al., 2005; Quesada et al., 1990), environmental niches in which their EPSs give them an advantage by enabling them to attach themselves to surfaces, improve nutrient acquisition and provide protection against environmental stress (Donlan & Costerton, 2002; Sutherland, 2001, 2002). Williams & Stewart (1994) suggested that a process of cell-to-cell signalling, or QS, plays a part in the regulation of surface attachment and biofilm maturation, leading logically to the idea that this QS system plays an important role in the capacity of halophilic bacteria to cope with their environment. In *H. anticariensis*, an EPS-producing species in which we have previously characterized the AHL signal molecules (Llamas et al., 2005), we obtained four AHL-deficient mutants. Two of them allowed us to characterize the hanR/hanI QS system involved in AHL production. Transcriptional analysis demonstrated that the *hanR* and *hanI* genes are on the same transcript, although *hanR* is expressed from its own promoter and appears to be independent of the AHLs produced by HanI (Tahrioui et al., 2011). To elucidate the mechanism by which the QS system in this halophilic bacterium is regulated we analysed the other two
AHL-deficient mutants and this led us to identify the gacS and gacA genes in *H. anticariensis*, which was the prime objective of this study.

A comparison between the predicted GacS and GacA proteins from a sequence analysis of *H. anticariensis* and their corresponding GacS and GacA homologues observed experimentally in other Gammaproteobacteria revealed relatively low identities. In fact the highest identity values corresponded to the described gene products, GacS and GacA, from the genomes of halophilic bacteria such as *H. elongata* and *C. salexigens* and from the genome drafts of other *Halomonas* species. Nevertheless, their biological functions in these halophilic bacteria have yet to be determined. The neighbour-joining phylogenetic trees resulting from the predicted GacS and GacA protein sequences of *H. anticariensis* both show that the sequences from the halophilic strains cluster together in a separate group from the *Pseudomonadaceae* and *Enterobacteriaceae* (Fig. S1), which concurs to a great extent with the topology of the GacA-protein-based tree found by de Souza et al. (2003). It is known that all TCSs share a modular domain organization (Wolanin et al., 2002; West & Stock, 2001), which we have also found in *H. anticariensis* FP35T. On the basis of the structural similarity between GacS and other multi-hybrid sensor kinases, as well as GacA and other response regulators, we propose that GacS is the cognate sensor of GacA in *H. anticariensis* FP35T.

AHL production by strain *H. anticariensis* FP35T depends upon overall regulation by GacS/GacA. A similar role for this TCS has also been reported in several other microorganisms, such as *Pseudomonas fluorescens* 2P24 (Yan et al., 2009); *Pseudomonas syringae* pv. tabaci (Marutani et al., 2008); *Pseudomonas syringae* (Quiñones et al., 2005); *Pseudomonas putida* (Bertani & Venturi, 2004) and *Serratia plymuthica* (Ovadis et al., 2004). Nevertheless, AHL production was not fully restored when the gacS gene was complemented in trans in *H. anticariensis* FP35T. The discrepancy found in correlating the AHL-defective phenotype shown by the gacS mutant with the corresponding complementation has been also reported in *Pseudomonas aeruginosa* where wild-type autoinducer levels were not restored in a TCS mutant complemented in trans (Dieppois et al., 2012). Since RT-PCR analysis revealed a considerable decrease in the expression of the QS genes *hanR* and *hanI* compared with the wild-type strain, it would seem that these two systems are interlinked. They may provide a way for *H. anticariensis* to combine information about surrounding cell density and respond appropriately to fluctuating environmental conditions.

We have also demonstrated that EPS production is under the positive control of the GacS/GacA TCS. This result is to be expected because the synthesis of EPS is a stationary-phase, gene-dependent process and the GacS/GacA TCS has been shown to regulate the expression of multiple phenotypes, including the production of virulence factors,

![Fig. 7. Complementation assays: (a) AHL production visualized on an agar plate by means of the *A. tumefaciens* NTL4 (pZLR4) indicator strain; (b) EPS production; (c) crystal-violet staining of 48 h biofilms.](a.png)
enzymes, antibiotics and polymers (Gooderham & Hancock, 2009). This regulation may take place directly, as it does in Azotobacter vinelandii, with GacA activating the synthesis of polymers, alginate and poly-β-hydroxybutyrate (PHB) by regulating the expression of rpoS, which in turn controls stationary-phase functions (Castañeda et al., 2000, 2001; Hernandez-Eligio et al., 2011). It has been shown recently that the RsmA in A. vinelandii represses post-transcriptionally the expression of PhbR, a transcriptional regulator that activates the phbBAC operon encoding the PHB-synthesis enzymes (Hernandez-Eligio et al., 2012). In the case of P. aeruginosa PA01, however, it has been suggested that the GacS/GacA TCS regulates the production of AHLs, which have a positive effect upon the induction of rpoS expression, which in turn activates genes expressed mainly during the stationary phase, such as those encoding the production of several virulence factors, including pyocyanine, exotoxin A, elastase and lipase enzymes and type III secretion system among others (Hogardt et al., 2004; Latifi et al., 1995; Reimann et al., 1997; Winson et al., 1995; Winzer et al., 2000). Furthermore, it has been reported that this signal transduction system in this bacterium acts exclusively by controlling the transcription of the RsmY and RsmZ regulatory small RNAs (Brencic et al., 2009). This second regulatory pathway may not be viable in H. anticariensis FP35T, however, since we have found that its EPS production is not controlled by AHLs (data not shown).

It is known that several sequential processes contribute to the formation of bacterial biofilms, including the initial attachment of the bacteria to surfaces in their environment, their aggregation into microcolonies and their secretion of EPSs and extracellular DNA and proteins (Flemming et al., 2007; Branda et al., 2005; Sutherland, 2001). Halomonas maura does not differ from this model, particularly in that EPS is essential to its formation of biofilms (Arco et al., 2005; Llamas et al., 2006). As mentioned above, the GacS/GacA TCS controls EPS biosynthesis in H. anticariensis, which prompted us to investigate whether this system affects biofilm formation. Importantly, biofilm formation was reduced in the H. anticariensis Gac mutants compared with the wild-type, which provides evidence that these two traits are correlated in this bacterium as they are in other micro-organisms. Lately, it has been reported that in Pseudomonas aeruginosa extracellular polymeric substances play a distinct key role during biofilm formation (Yang et al., 2011) and this development is controlled by TCSs among other elements such us QS, c-di-GMP and sigma factors (Mikkelsen et al., 2011). Since biofilm formation is a complex process controlled by multiple factors, the TCS might indirectly affect it by contributing to the regulation of some key genes, all of which requires further study.

In summary, this study provides some initial insights into the phenotypes controlled by the GacS/GacA TCS in the halophilic bacterium H. anticariensis FP35T, such as AHL synthesis, EPS production and biofilm formation during the stationary growth phase. In order to throw further light on the potential functional importance of this system, a full genome analysis is now being undertaken to reach a comprehensive appreciation of gene-expression regulatory control mediated by the GacS/GacA TCS in H. anticariensis FP35T.

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