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Quorum sensing in halophilic bacteria: detection of *N*-acyl-homoserine lactones in the exopolysaccharide-producing species of *Halomonas*

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Abstract Some members of the moderately halophilic genus *Halomonas*, such as *H. eurihalina, H. maura, H. ventosae* and *H. anticariensis*, produce exopolysaccharides with applications in many industrial fields. We report here that these four species also produce autoinducer molecules that are involved in the cell-to-cell signaling process known as quorum sensing. By using the *N*-acyl homoserine lactone (AHL) indicator strains *Agrobacterium tumefaciens* NTL4 (pZRL4) and *Chromobacterium violaceum* CV026, we discovered that all the *Halomonas* strains examined synthesize detectable AHL signal molecules. The synthesis of these compounds was growth-phase dependent and maximal activity was reached during the late exponential to stationary phases. One of these AHLs seems to be synthesized only in the stationary phase. Some of the AHLs produced by *H. anticariens* FP35^T were identified by gas chromatography/mass spectrometry and electrospray ionization tandem mass spectrometry as *N*-butanoyl homoserine lactone (C_4-HL), *N*-hexanoyl homoserine lactone (C_6-HL), *N*-octanoyl homoserine lactone (C_8-HL) and *N*-dodecanoyl homoserine lactone (C_{12}-HL). This study suggests that quorum sensing may also play an important role in extreme environments.

Keywords Quorum sensing · *N*-acyl-homoserine lactone · Exopolysaccharide · Halophiles · *Halomonas*

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Introduction

Quorum sensing is a population-density dependent gene-expression mechanism which involves the production of signal molecules known as autoinducers (González and Marketon 2003; Whitehead et al. 2001), a phenomenon which is being extensively studied in bacteria that live in pathogenic or symbiotic associations (Fuqua et al. 1994; Kleerebezem et al. 1997). The autoinducer signals from gram-negative Proteobacteria are generally *N*-acyl homoserine lactones (AHLs) which differ in the length and substitution of their respective acyl side chains, conferring upon them signal specificity (Fuqua et al. 1995; Hwang et al. 1995). One of the most thoroughly studied quorum-sensing systems is found in the luminescent bacterium *Photobacterium fischeri* (formerly *Vibrio fischeri*), a symbiont of various marine hosts (Engebrecht et al. 1983; Engebrecht and Silverman 1984; Kaplan and Greenberg 1985; Lupp et al. 2003). In this model system, an autoinducer synthase, LuxI, is responsible for the production of 3-oxohexanoyl homoserine lactone (3-oxo-C_6-HL). As bacterial population density increases during interaction with the host, the diffusible AHL molecules accumulate and upon reaching a particular threshold level, they bind and activate LuxR, a transcriptional activator. LuxR then induces expression of the *lux* operon, which includes the *luxI* gene, leading to the production of more AHLs (Eberhard et al. 1991) and the expression of the genes responsible for luminescence (Fuqua et al. 1994; Swift et al. 1999).

Bacterial cell functions regulated by quorum sensing include the expression of virulence factors and exoenzymes in *Pseudomonas aeruginosa* and *Erwinia carotovora* (Beck Von Bodman et al. 2003; de Kievit and

Iglewski 2000), conjugal DNA transfer and plasmid copy number control in *Agrobacterium tumefaciens* (Farrand 1998; Fuqua et al. 2001; Fuqua et al. 1994), production of antibiotics in *Chromobacterium violaceum* (McClean et al. 1997), biofilm formation in *P. aeruginosa* and *Streptococcus gordonii* (Davies et al. 1998; McNab et al. 2003) and exopolysaccharide production in *Pantoea stewartii* (Beck Von Bodman et al. 1998) and *Sinorhizobium meliloti* (Marketon et al. 2003).

One significant group of organisms for which quorum sensing has not been previously reported, however, is the moderate halophiles. The genus *Halomonas*, part of the γ-subclass of the Proteobacteria, includes moderately halophilic, gram-negative bacteria characteristically showing the best growth in media containing 0.5–2.5 M NaCl and exhibiting an extraordinary ability to rapidly adapt to changes in the external salt concentration (Kushner and Kamekura 1988). Their presence is widespread in salterns, saline soils, seawater, and marshes where they constitute one of the most abundant families of halophilic bacteria and they are believed to exert a considerable influence within their ecological niches (Oren 2002; Ventosa et al. 1998). Taxonomic and phenotypic studies have suggested that the moderate halophiles do form part of larger microbial communities (reviewed in Ventosa et al. 1998).

Over the last decade, interest in the *Halomonas* species has centered on their ability to produce exoenzymes, exopolysaccharides and other commercially valuable products (Oren 2002; Quesada et al. 2004; Rodríguez-Valera et al. 1991; Ventosa et al. 1998; Ventosa 2004; Vreeland 1993). Currently, the genus *Halomonas* includes about thirty species of halophilic bacteria, most of which have been isolated from saline environments (Dobson and Franzmann 1996; Mata et al. 2002; Ventosa et al. 1998; Vreeland et al. 1980). Four of these species, *H. eurihalina*, *H. maura*, *H. ventosae* and *H. anticariensis*, isolated from the rhizosphere of xerophytic plants, have been identified as having the ability to produce large quantities of exopolysaccharides with novel physical and chemical characteristics (Arias et al. 2003; Béjar et al. 1998; Bouchotroch et al. 1999; Bouchotroch et al. 2000; Bouchotroch et al. 2001; Martínez-Cánovas et al. 2004a, b; Quesada et al. 1990; Quesada et al. 2004). How the biosynthesis of these exopolysaccharides is regulated and whether their production responds to environmental factors and/or population-density remains to be investigated.

We show here that all four exopolysaccharide-producing species of *Halomonas* that we examined synthesize AHLs which can be detected by the commonly used indicator organisms, *A. tumefaciens* NTL4 (pZLR4) and *C. violaceum* CV026. These AHLs are produced in a typical population-density dependent manner with maximal production achieved during the exponential to stationary growth phases. In addition, using gas chromatography/mass spectrometry (GC/MS) and electrospray ionization tandem mass spectrometry (ESI MS/MS), we have identified four AHLs produced by one of the *Halomonas* strains. To our knowledge, this is the first report describing the production of quorum-sensing signal molecules in the moderately halophilic bacteria. Careful analysis of the *Halomonas* quorum-sensing system may provide insights into the role that cell-to-cell communication may play in bacteria that grow in moderate-to-high salt environments.

Materials and methods

Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. *H. maura* was isolated from a solar saltern in Asilah, Morocco (Bouchotroch et al. 1999; Bouchotroch et al. 2001) and *H. eurihalina, H. ventosae* and *H. anticariensis* were isolated from saline soils in Alicante, Jaén and Málaga in southern Spain (Martínez-Cánovas et al.

Table 1 Bacterial strains used in this work

Strain	Relevant characteristics	Reference
Halomonas eurihalina strains		
F2-7	Isolated in Alicante, Spain	Quesada et al. 1990)
F9-6^T (=ATCC 49336^T)	Isolated in Alicante, Spain	Quesada et al. (1990)
M4	Isolated in Almería, Spain	Quesada et al. (2004)
Halomonas maura strains		
S-30	Isolated in Asilah, Morocco	Bouchotroch et al. (2001)
S-31^T (=CECT 5298^T)	Isolated in Asilah, Morocco	Bouchotroch et al. (2001)
X-2	Isolated in Souk El Arbaa, Morocco	Quesada et al. (2004)
B-100	Isolated in Murcia, Spain	Quesada et al. (2004)
Halomonas ventosae		
Al-12^T (=CECT 5797^T)	Isolated in Jaén, Spain	Martínez-Cánovas et al. (2004b)
Halomonas anticariensis		
FP34	Isolated in Málaga, Spain	Martínez-Cánovas et al. (2004a)
FP35^T (=CECT 5854^T)	Isolated in Málaga, Spain	Martínez-Cánovas et al. (2004a)
FP36	Isolated in Málaga, Spain	Martínez-Cánovas et al. (2004a)
Indicator strains		
Agrobacterium tumefaciens NTL4 (pZLR4)	NT1 derivate carrying a *traG*::*lacZ* reporter fusion	Luo et al. (2001)
Chromobacterium violaceum CV026	CV017 derivate containing *cviI*::Tn*5xylE*	McClean et al. (1997)

2004a, b; Quesada et al. 1990; Quesada et al. 1993). All four strains were grown at 32°C on MY medium (10 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone no. 3) (Moraine and Rogovin 1966) plus 7.5% v/w marine salts (Rodríguez-Valera et al. 1981). *A. 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 (11 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 mg biotin, 27.8 mg $CaCl_2$ and 246 mg $MgSO_4$ per liter) containing 50 µg of gentamycin per ml. *C. violaceum* CV026 was grown at 30°C in LB medium.

Preparation and TLC analysis of crude AHL extracts

AHL molecules were extracted following the technique described by Marketon et al. (2002). Briefly, 10 ml cultures were grown to early stationary phase (optical density of approximately 2.8 at 600 nm) and extracted twice with equal volumes of dichloromethane. The extracts were dried and resuspended in 20 µl of 70% v/v methanol. The AHLs were analyzed on reverse-phase C_{18} thin-layer chromatography (RP-C_{18} TLC) using methanol:water 7:3 (v/v) as the mobile phase. Once the plates were dry, they were overlaid with top agar containing the indicator organism and incubated overnight at 30°C. For the *A. tumefaciens* NTL4 (pZLR4) overlay, a 6–8 h culture in MGM medium was mixed with an equal volume of fresh medium, 1.5% w/v Bacto Agar and 80 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. For the *C. violaceum* CV026 overlay, a 4–6 h culture in LB medium was mixed with an equal volume of 0.75% w/v Bacto Agar.

AHL standards were obtained from Sigma [*N*-(β-Ketocaproyl)-L-homoserine lactone (3-oxo-C_6-HL)] and Fluka [*N*-butyryl-DL-homoserine lactone (C_4-HL), *N*-hexanoyl-DL-homoserine lactone (C_6-HL), *N*-octanoyl-DL-homoserine lactone (C_8-HL) and *N*-dodecanoyl-DL-homoserine lactone (C_{12}-HL)]. The C_4-HL and the 3-oxo-C_6-HL comigrate under the conditions of our TLC analysis.

AHL purification and identification

A 2.5 liter culture of *H. anticariensis* FP35^T was grown in MY broth medium with 7.5% w/v NaCl to stationary phase (optical density of 2.8 at 600 nm) and the whole culture extracted twice with equal volumes of dichloromethane. The extract was dried by rotary evaporation and resuspended in 5 ml of dichloromethane, after which a 5 µl aliquot was tested for activity by TLC overlay analysis as described above. The remaining methylene chloride extract was redissolved in 1 ml of chloroform and 5 ml of ethanol was added. The resulting precipitate was removed by centrifugation and the supernatant was evaporated to dryness. The residue was redissolved in 1 ml of chloroform and loaded onto a solid phase extraction amino column (Phenomenex) that had been pretreated with hexane followed by chloroform. The column was eluted with 20 ml of chloroform/2-propanol 2:1 (v/v). After evaporation of the eluate, the residue was taken up in 0.5 ml of acetonitrile:water 3:1 (v/v), centrifuged and the supernatant subjected to HPLC using a 1×25 cm reverse phase C_8 Column (Phenomenex). The column was eluted at 3 ml/min with 10% v/v acetonitrile/water for 2 min, then a gradient to 100% v/v acetonitrile over 20 min, followed by 100% v/v acetonitrile for another 12 min. Fractions (6 ml) were collected. After evaporation of the fractions using a Speed Vac, the residues were redissolved in 10 µl of ethyl acetate and analyzed by GC/MS using 0.2 µl per spectrum. The ethyl acetate was allowed to evaporate, and the residues were redissolved in 150 µl of acetonitrile, then 50 µl of 1% v/v formic acid in water was added, the fractions centrifuged and analyzed using ESI MS/MS.

Gas chromatography/mass spectrometry analysis was performed with a Hewlett Packard Series II model 5890 GC fitted with a 5971A Mass Selective Detector and using a 12 m ×0.2 mm column of 5% PH ME Siloxane (Agilent). All fractions were first tested with selective ion monitoring (SIM) using mass 143, a fragment formed by all AHLs through a McLafferty Type II mechanism involving the amide carbonyl group (McLafferty 1966). The fractions were then tested in scan mode to obtain the fragmentation patterns of the peaks highlighted in the previous SIM run. The retention times and fragmentation patterns were compared to those of the authentic compounds to make an identification. Under our conditions, 3-oxo-AHLs decomposed in the injector of the GC to give the methyl ketones with a carbon number one less than that of the acyl groups of the 3-oxo-AHLs. The retention times and fragmentation patterns of authentic methyl ketones were compared to those of peaks from the HPLC fractions, yielding a tentative identification; positive identification of a 3-oxo-AHL was made only after ESI MS/MS.

ESI MS/MS was performed as described (Marketon et al. 2002). In ESI MS/MS, all AHLs were found to fragment in such a way as to give a positive ion of mass 102 (a protonated homoserine lactone) and a neutral acyl fragment, and to give a neutral homoserine lactone of mass 101 and a positive acyl fragment. By looking for the precursor ions of these fragments having masses corresponding to those of AHLs having acyl groups that may be expected to be derived from fatty acid biosynthesis (Moré et al. 1996), we were able to make a tentative AHL identification, which was then confirmed through their fragmentation patterns when compared to those of the corresponding authentic compounds.

Growth phase dependent analysis of AHL production in *H. anticariensis* strain FP35^T

Cultures of *H. anticariensis* strain FP35^T were grown overnight at 32°C in MY broth medium with 7.5% w/v

NaCl. The cells were washed and resuspended in an equal volume of 0.85% w/v NaCl. We then inoculated 2.5 ml of the cell resuspension into a 1 liter flask containing 250 ml of MY medium and incubated at 32°C for 12 h. Samples (2.5 ml) from this culture were collected at different stages over a period of 12 h and AHLs were extracted and separated by reverse phase-C_{18} TLC chromatography as described above. Once the plates were dry, they were overlaid with top agar containing the *A. tumefaciens* NTL4 (pZLR4) indicator strain.

Results and discussion

Detection of autoinducer molecules in exopolysaccharide-producing *Halomonas* species

Most of the quorum-sensing systems characterized to date are found in bacteria that establish relationships, either pathogenic or symbiotic, with plant or animal hosts. AHL production is also present in the marine bacterial genus *Roseobacter*, which forms aggregates (marine snow) in the ocean (Gram et al. 2002). More recently, it was reported that the haloalkaliphilic archaeon *Natronococcus occultus* is capable of producing autoinducer molecules, which might control the synthesis of an extracellular protease (Paggi et al. 2003). The two latter cases are examples of the role that quorum sensing may play in bacterial communal interactions. In the moderately halophilic bacteria such as *Halomonas*, no quorum-sensing system has been described to date. In our efforts to explore the regulation of exopolysaccharide production and other processes in the *Halomonas* species, we decided to determine whether members of this genus possessed a population-density dependent gene regulation system. In order to attain this aim, we screened eleven exopolysaccharide-producing *Halomonas* strains (Table 1) belonging to the species *H. eurihalina*, *H. maura, H. ventosae* and *H. anticariensis*. Two indicator organisms, which respond to different sizes of AHLs, *A. tumefaciens* NTL4 (pZLR4) and *C. violaceum* CV026, were used to detect the presence of quorum-sensing signal molecules in *Halomonas* cultures. NTL4 (pZLR4) is unable to produce its own AHLs and contains a *lacZ* fusion to the quorum-sensing regulated gene *traG*. This strain is sensitive to AHLs with medium-to-long acyl chains, which when added exogenously, result in the activation of the *lacZ* fusion, which is detectable by the production of a blue color in the presence of X-Gal (Shaw et al. 1997). The CV026 strain is a mutant unable to produce its own quorum-sensing signal molecules and it responds to exogenously added AHLs with short-to-medium acyl chains by producing a pigment called violacein (McClean et al. 1997).

To determine whether bacteria belonging to the *Halomonas* genus synthesize AHLs, we examined each strain by cross streaking against the NTL4 (pZLR4) and CV026 indicators on either LB or MY plates. This proved to be difficult since we could not find a compatible salt concentration at which both the indicator organisms and all the *Halomonas* strains would grow efficiently. Only the *Halomonas* strains, *H. maura* and *H. anticariensis* grew well and activated the NTL4 (pZLR4) (Fig. 1a) and CV026 (Fig. 1b) indicator strains in LB/MC medium with 1% w/v NaCl.

Characterization of the AHLs produced by *Halomonas*

The use of the indicator organisms in combination with thin-layer chromatography (TLC) provides a simple and rapid way of determining the number and nature of the AHLs produced by a particular strain (Shaw et al. 1997). Culture extracts of the different *Halomonas* strains (Table 1), contained at least two autoinducer molecules detectable with the NTL4 (pZLR4) indicator (Fig. 2a, Table 2). However, only the three *H. anticariensis* strains synthesized AHLs in sufficient quantities to activate the CV026 indicator (Fig. 2b). It should be noted that the *H. anticariensis* strains produce about five times more AHL than the rest of the *Halomonas* strains assayed (5 µl of their extracts were used for detection by

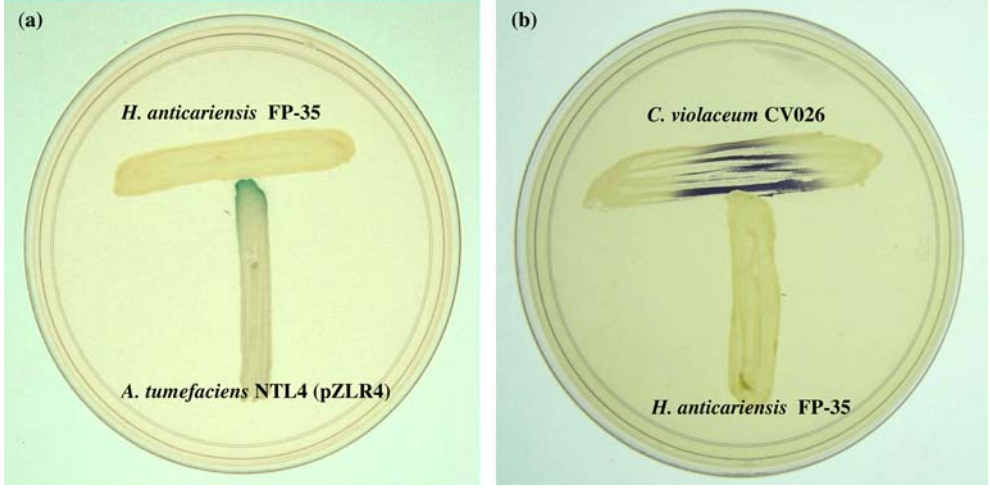


Fig. 1 Detection of AHL production in *Halomonas*. **a** *H. anticariensis* FP35^T streaked next to *A. tumefaciens* NTL4 (pZLR4) on LB/MC agar plates containing 80 µg ml^{-1} of X-Gal. **b** *H. anticariensis* FP35^T streaked next to *C. violaceum* CV026 on LB/MC agar plates

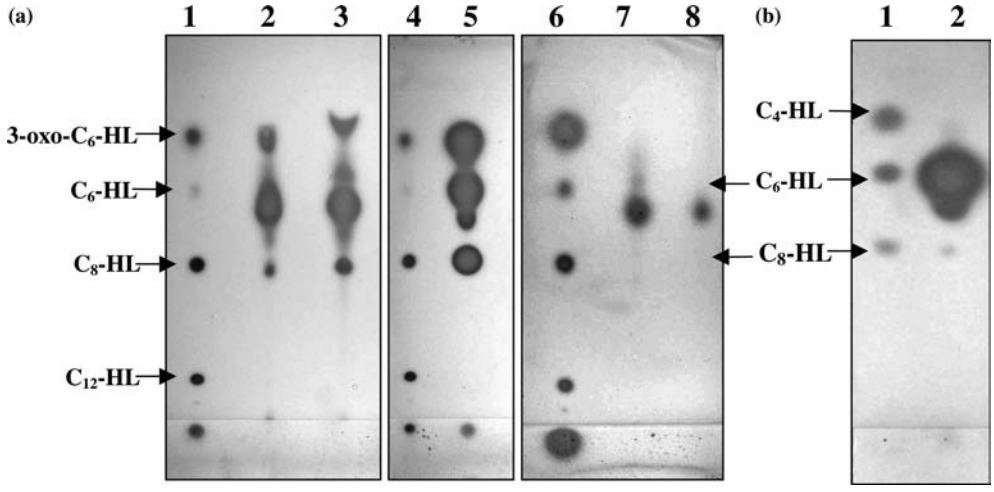


Fig. 2 Comparison of the AHL profiles from the different *Halomonas* species on a reverse-phase C_{18} thin-layer chromatography. **a** Plate overlaid with the NTL4 (pZLR4) indicator strain. *Lanes 1* and *4,* synthetic AHL standards, 3-oxo-C_6-HL (4.7 pmol), C_6-HL (804 pmol), C_8-HL (31.6 pmol) and C_{12}-HL (2.4 nmol); *lane 6*, synthetic AHL standards, 3-oxo-C_6-HL (7 pmol), C_6-HL (1.2 nmol), C_8-HL (47.4 pmol) and C_{12}-HL (3.6 nmol); *lane 2, H. eurihalina* F2-7; *lane 3*, *H. maura* S-31^T; *lane 5, H. anticariensis* FP35^T; *lane 7, H. ventosae* Al-12^T; *lane 8,* uninoculated MY 7.5% w/v NaCl broth medium. Each lane contains 20 µl of extract except for lane 5, which contains 5 µl. **b** Plate overlaid with the CV026 indicator. *Lane 1,* C_4-HL (7 nmol), C_6-HL (80.4 nmol), C_8-HL (3 nmol) standards; *lane 2,* 10 µl of *H. anticariensis* FP35^T extract

NTL4 (pZLR4) instead of 20 µl needed for the rest of the *Halomonas* species). A sample prepared from 10 ml of uninoculated MY broth medium containing 7.5% w/v NaCl showed a very slight induction of the NTL4 (pZLR4) indicator strain (Fig. 2a, lane 8), but did not activate the CV026 indicator (data not shown). This type of background has been attributed to the presence of signal compounds generated during the sterilization of the media (Holden et al. 1999).

The analysis of the AHL extracts from each strain by TLC showed that all of the strains belonging to the same species had a similar AHL profile when analyzed by the NTL4 (pZLR4) indicator organism (data not shown and Table 2). The *H. ventosae* species produced lower levels of AHLs than the rest of the bacteria tested. The *H. eurihalina* and *H. maura* species have similarities in their AHL production patterns which could be attributed to the fact that they are taxonomically closely related (Bouchotroch et al. 2001). Both microorganisms synthesized at least three NTL4 (pZRL4) detectable AHLs, with mobilities similar to that of the C_8-HL, C_6-HL and 3-oxo-C_6-HL/C_4-HL standards (Fig. 2A, lane 2 and 3 and Table 2). These three signal molecules were also produced by *H. anticariensis* strains, although this species synthesized about five times more AHLs than any of the other halophilic bacteria examined.

The pattern of AHL spots from *H. anticariensis* detected by the two indicator organisms was slightly different. The CV026 indicator strain, which is more sensitive to AHLs with short-to-medium acyl chains, showed a different AHL pattern from the one observed with NTL4 (pZLR4) indicator. Four of the *H. anticariensis* FP35^T signal molecules were detected with NTL4 (pZLR4) (Fig. 2a, lane 5), while only two of these autoinducer signals were detected with the CV026 indicator (Fig. 2b, lane 2). The CV026 indicator detected only

Table 2 Detection of AHL patterns in the *Halomonas* species by using TLC and indicator organism

Strain	Number of spots	AHL identification according to the migration in the TLC
H. eurihalina strains		
F2-7	3	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
F9-6^T	3	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
M4	3	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
H. maura strains		
S-30	3–4	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
S-31^T	3–4	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
X-2	3–4	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
B-100	3–4	C_8-HL (+), C_6-HL (++), 3-oxo-C_6-HL/C_4-HL (+)
H. ventosae		
Al-12^T	1–2	ND
H. anticariensis strains		
FP-34	3–4	C_8-HL (++), C_6-HL (+++), 3-oxo-C_6-HL/C_4-HL (+++)
FP-35^T	3–4	C_8-HL (++), C_6-HL (+++), 3-oxo-C_6-HL/C_4-HL (+++)
FP-36	3–4	C_8-HL (++), C_6-HL (+++), 3-oxo-C_6-HL/C_4-HL (+++)

ND not determined
(+, ++, +++) signal molecule approximated amount

AHLs (Fig. 2b) with mobility similar to that of the C_8-HL and C_6-HL standards. There also was a third signal molecule produced in low quantities, which migrated close to the 3-oxo-C_6-HL/C_4-HL standards (data not shown). The differences found in the pattern and amount of AHLs produced by *H. anticariensis* species with regard to the rest of the *Halomonas* species studied could be attributed to the differences in their phenotypic, phylogenetic and chemotaxonomic characteristics. In fact, *H. anticariensis* strains form a clearly separated group (Martínez-Cánovas et al. 2004a).

AHL synthesis in *H. anticariensis* FP35^T is growth-phase dependent

Most of the organisms that harbor quorum-sensing systems produce a higher concentration of autoinducer molecules during their late exponential or early stationary phases (Cha et al. 1998; Pearson et al. 1994; Shaw et al. 1997). To determine the pattern of AHL production in *Halomonas*, we selected the high AHL producing strain, *H. anticariensis* FP35^T. AHL molecules were extracted from the FP35^T culture at different stages over a period of 10 h (Fig. 3a) and analyzed by reverse phase-C_{18}-TLC. The AHL production pattern during growth, detected by the NTL4 (pZLR4) indicator strain, is shown in Fig.3b. The highest concentration of AHLs occurred at high cell densities ($OD_{600} = 2.5$) (Fig. 3, lane 7). We were able to detect a few AHLs at densities as low as 1.3 (Fig 3b, lane 3). The first detectable autoinducer compounds, corresponding to the signal molecules with mobilites similar to those of the C_8-HL and C_6-HL standards (Fig. 3b, lane 3), began to be synthesized after 6 h of growth. A third compound, with a mobility close to that of the 3-oxo-C_6-HL/C_4-HL standards, was produced after 7 h of growth ($OD_{600} = 1.8$) (Fig. 3b, lane 4). These three signal molecules continued to accumulate with growth and remained during the stationary phase, as shown in Fig. 2a (lane 5) and Fig. 3b (lane 7). It should be noted that a fourth AHL, which migrates between the C_6-HL and C_8-HL standards, appears to be synthesized only in the stationary phase (Fig. 3b, lane 7, Fig. 2a, lane 5). An analysis of the AHL pattern in *H. anticariensis* FP35^T at different cell densities suggests that the production of some of these autoinducer molecules may be inhibited until a high cell density is reached, as occurs in the *tra* quorum-sensing system in *A. tumefaciens* (Fuqua et al. 1995; Hwang et al. 1995).

H. anticariensis FP35^T synthesizes C_4-HL, C_6-HL, C_8-HL and C_{12}-HL

To determine the nature of the signal molecules produced by *H. anticariensis* FP35^T, a large culture of this organism was grown and extracted twice with dichloromethane (see Materials and Methods). This

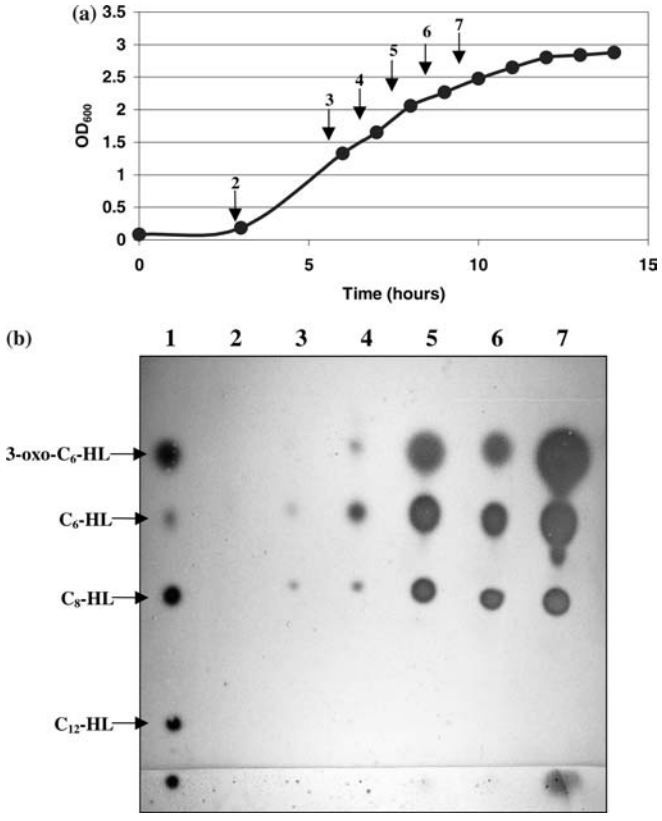
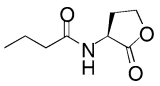
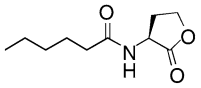
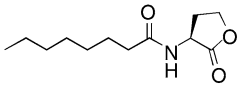
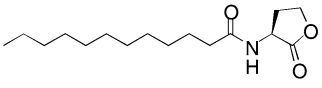


Fig. 3 Analysis of the AHLs produced by *H. anticariensis* FP35^T during different growth phases. **a** Growth of *H. anticariensis* FP35^T strain at 32°C in MY medium with 7.5% w/v NaCl. Cell growth was monitored by measuring the optical density at 600 nm. **b** Samples taken from a culture at different stages during its growth (see Fig. 3a) were chromatographed on a reverse-phase C_{18} thin-layer plate and overlaid with the NTL4 (pZLR4) indicator strain. *Lane 1*, synthetic AHL standards, 3-oxo-C_6-HL (4.7 pmol), C_6-HL (804 pmol), C_8-HL (31.6 pmol) and C_{12}-HL (2.4 nmol). *Lanes 2–7*, 5 µl of *H. anticariensis* FP35^T extracts obtained from cultures grown to $OD_{600} = 0.05$, 0.187, 1.3, 1.8, 2.0, 2.2, and 2.5 respectively

extract was separated on a reverse phase C_8-HPLC column and each fraction was then tested for activity with the NTL4 (pZLR4) indicator strain. All fractions were analyzed by gas chromatography/mass spectrometry (GM/MS) and electrospray ionization tandem mass spectrometry (ESI MS/MS) to identify their AHL structures.

The mass spectral analysis revealed that strain FP35^T synthesizes four unsubstituted AHLs (Table 3) while no signals were detected from a sample of uninoculated media. A short acyl-chain AHL, C_4-HL, is produced by FP35^T in low quantities (about tenfold less that the C_6-HL) and it can be only weakly detected by the *C. violaceum* CV026 indicator (data not shown). Two medium acyl-chain AHLs, C_6-HL and C_8-HL, were also detected in the extracts. The C_6-HL is the most abundant AHL produced by the FP35^T strain, while the C_8-HL is present at a concentration of about fivefold less than the C_6-HL. A long acyl-chain AHL, C_{12}-HL, was found to be produced in small quantities (similar to those of the

Table 3 Identification by ESI MS/MS of four AHLs produced by *Halomonas anticariensis* FP35^T

Proposed structure	Name
	***N*-(tetrahydro-2-oxo-3-furanyl)-butanamide (C_4-HL)**
	***N*-(tetrahydro-2-oxo-3-furanyl)-hexanamide (C_6-HL)**
	***N*-(tetrahydro-2-oxo-3-furanyl)-octanamide (C_8-HL)**
	***N*-(tetrahydro-2-oxo-3-furanyl)-dodecanamide (C_{12}-HL)**

C_4-HL). This component was only marginally detected by the NTL4 (pZLR4) indicator strain.

The observation that the *Halomonas* species produce growth-phase-dependent *N*-acyl homoserine lactones will focus our future work on the examination of whether these signal molecules might regulate cellular process such as the production of exopolysaccharides, exoenzymes, and/or biofilm formation in these bacteria.

Our research group has reported that exopolysaccharide production in *Halomonas* starts in the late exponential phase and reaches a maximum during the stationary phase (Quesada et al. 2004). These observations suggest that EPS synthesis might be induced in response to high population density, and therefore, could be regulated by quorum-sensing based signaling. In fact, exopolysaccharide production in some bacteria, such as *Pantotea stewartii* (Beck Von Bodman et al. 1998) and *Sinorhizobium meliloti* (Marketon et al. 2003), has already been described as being controlled by quorum sensing. To explore this possibility in *Halomonas*, we compared the EPS levels produced over a 5-day period by *H. anticariensis* FP35^T in the presence and absence of additional AHLs in the culture. We observed in this preliminary experiment a small but consistent increase in overall EPS production in the culture supplemented with additional AHLs (data not shown). This increase could suggest a role for quorum sensing in *H. anticariensis* EPS production. Confirmation of these results awaits the isolation of quorum-sensing mutants. This work is currently underway.

It is becoming increasingly clear that population-density-mediated gene expression is widespread in bacteria involved in pathogenic and symbiotic associations. Recent reports (Gram et al. 2002; Johnson et al. 2005; Paggi et al. 2003), including this study, suggests that it may also play an important role in extreme environments within the bacterial communities that populate them. The characterization of global regulatory systems in the *Halomonas* species, such as a quorum-sensing system, may provide information on their unique ability to adapt to a wide range of hypersaline habitats.

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