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SHORT COMMUNICATION

Identification of N-Hexadecanoyl-L-homoserine lactone (C16-AHL) as signal molecule in halophilic bacterium *Halomonas smyrnensis* AAD6

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Abstract Quorum sensing (QS) mechanisms regulate a variety of vital functions such as motility, growth inhibition, biofilm development, virulence expression and plasmid conjugation. Many of these activities are essential for the well-being of organisms, in particular in extreme conditions such as hypersaline environments. Halophiles are able to synthesize products that are stable, and to exploit their activities under such "extreme" conditions, namely saline environments such as marine habitat, salt lakes, brines and saline soils. The versatility of these products leads researchers to address their interest in biotechnological applications. In this study, the QS activity of *Halomonas smyrnensis* AAD6—a moderately halophilic and exopolysaccharide-producing bacterium—was investigated. Dichloromethane extracts from stationary phase cultures of *Halomonas smyrnensis* AAD6 were partially purified, and C_{16}-AHL was identified by LC-MS analysis. The synthesis of autoinducers was assayed and their production was detected starting from 48 h of growth. The growth-phase dependent production of exopolysaccharide (EPS) and signal molecules suggested that these activities could be linked.

Keywords *Halomonas smyrnensis* · AHL · *Agrobacterium tumefaciens* NTL4 · Quorum sensing · UPLC-MRM analysis

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Findings

Microbial communities coordinate gene expression at high cell density through a mechanism known as quorum sensing (QS). The QS mechanism basically relies on the synthesis of small diffusible molecules (termed "autoinducers") that bacteria release into the environment where they accumulate. When the amount of signal molecules reaches a critical concentration, the bacterial communities react to the reached "quorum" by co-ordinating gene expression that results in phenotypic outcomes (Fuqua et al. 1994). A general distinction can be made between Gram-negative bacteria, in which gene expression is induced mainly by N-acyl homoserine lactones (AHLs), and Gram-positive bacteria, which synthesize mainly peptides as signalling systems (Abbamondi et al. 2014; Tommonaro et al. 2012). Signal molecule production is an auto-regulated mechanism where the target genes activated by QS often incorporate those required for the synthesis of the autoinducers, thus the signal is auto-amplified (Williams 2007; Atkinson and Williams 2009).

Physiological activities linked to QS systems are various, and include biofilm development, virulence expression, plasmid conjugation, motility and growth inhibition, all of which are associated with a variety of physiological functions that sustain life (Williams 2007; Rader et al. 2007).

Many of these activities are required for the well-being of organisms in extreme conditions, thus it is possible to speculate that QS-regulated gene expression could be part of the strategy evolved by bacteria and archaea to thrive in extreme sites. The term "extreme" is adopted to describe a habitat in which physical or chemical parameters are prohibitive for the majority of organisms, such as temperature, pH, salt concentration and hydrostatic pressure. Halophiles are salt-loving organisms living in diverse environments where high salt concentrations are found. According to their requirement for salt

concentration, halophiles are classified into: slight (2–5 %NaCl), moderate (5–20 % NaCl) and extreme (20–30 % NaCl) halophiles. (DasSarma and DasSarma 2012; Saum and Muller 2008).

In the last two decades, interest in the biotechnological applications of halophiles has grown. Halophilic microorganisms produce several biomolecules (exopolysaccharide, stable enzymes) with great potential for biotechnology (DasSarma et al. 2010; Margesin and Schinner 2001; Di Donato et al. 2011). QS mechanisms could affect the production of several biomolecules, thus a deeper study of halophiles and their chemical communication mechanisms may help improve the efficiency of biotechnological processes (Mangwani et al. 2015; Visick and Fuqua 2005).

QS systems mediated by AHLs are widespread within *Halomonadaceae*. In an extensive study performed on 43 strains of that family, all the analyzed strains were found to activate an *Agrobacterium tumefaciens* NTL4 (pZLR4) bioreporter that is sensitive to AHLs with medium to long acyl chains. Further analysis by PCR and DNA sequencing approaches demonstrated that most of the studied species contained LuxI homolog genes. A thin layer chromatography (TLC)-overlay test was also performed, and C_6-AHL was identified as the most predominant AHL molecule. The isolation and chemical characterization of C_6-AHL from *Halomonas anticariensis* FP35^T by means of chromatography/mass spectrometry and electrospray ionization tandem mass spectrometry (ESI MS/MS) confirmed the previous results (Llamas et al. 2005; Tahrioui et al. 2013).

In this study, the main aim was to identify the signal molecule responsible for QS activity detected in cell-free medium extract of *Halomonas smyrnensis* AAD6, a moderately halophilic and exopolysaccharide (EPS)-producing bacterium (Poli et al. 2013). QS activity was detected starting from $t=48$ h of growth. QS activity was found to be mediated by AHLs and, in particular, C_{16}-AHL was identified by LC-MS/MS analysis in cell-free medium extracts of *H. smyrnensis* AAD6 strain. The strain *H. smyrnensis* AAD6 was isolated and grown as reported in Poli et al. (2009). Briefly, a Certomat BS-1 orbital shaker (Braun, Melsungen, Germany) set at 37 °C and 180 rpm agitation rate was used for the cultivations, and the composition of the growth medium was (g L^{-1}): 137.2 g NaCl; 50 g sucrose; 7 g K_2HPO_4; 2 g KH_2PO_4; 0.1 g $MgSO_4.7H_2O$; 1 g $(NH_4)_2SO_4$ and 0.5 g peptone. Trace element solution (per 100 mL): $MnCl_2{\cdot}4H_2O$ 36 mg, $ZnSO_4{\cdot}7H_2O$ 44 mg, $FeSO_4{\cdot}7H_2O$ 230 mg, $CuSO_4{\cdot}5H_2O$ 5 mg was filter sterilized and added as 0.1 % (v/v) to the medium. Filter sterilized thiamine solution was added at 0.8 mg L^{-1} final concentration. Cell growth was monitored by measuring the optical densities (OD) at 660 nm using Lambda35 UV/Vis spectrophotometer (Perkin Elmer, Waltham, Massachusetts), and OD values were converted to dry cell weight (DCW) by means of a calibration chart as described previously (Poli et al. 2009). To determine the EPS content at certain time intervals, samples collected from the fermentation broth were centrifuged at 10,000 rpm for 20 min to precipitate cells. The supernatant phases were treated with an equal volume of ethanol and kept overnight at −18 °C. The precipitate from the ethanol dispersion was collected by centrifugation at 12,000 rpm for 30 min, redissolved in hot distilled water, dialyzed against several runs of distilled water for 3 days, and then lyophilized. The dry weight of the EPS samples was determined and used for yield calculations.

To detect the production of signal molecules, spent media (1 L) from shaking cultures of *H. smyrnensis* AAD6 at different times (0, 24, 48, 72 and 96 h) were centrifuged at 10,000 rpm for 40 min. Pellets were stored at −20 °C for further investigation, while supernatants were extracted with dichloromethane (1:1 v/v; twice). Dichloromethane extracts were dried under vacuum at T < 40 °C and dissolved in acetonitrile.

Supernatant extracts (2 mg) and standards (3-oxo-C_6-AHL 10 μM and 3-oxo-C_{10}-AHL 400 μM) were applied to RP C-18 thin-layer chromatography (TLC) plates (20 × 20 cm; VWR International), and a mobile phase of 60 % (*v/v*) aqueous methanol was used to separate the extracts. The TLC plates were overlaid with 100 mL of AGTN (Tempé et al. 1977) soft agar (0.5 % w/v) supplemented with 0.5 % glucose, 40 μg mL^{-1} X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside), antibiotics (streptomycin, 50 μg mL^{-1} and tetracycline, 4 μg mL^{-1}) and the biosensor *A. tumefaciens* NTL4 (pCF218; pCF372) (Fuqua and Winans 1996). The TLC plates were kept in a sterile container and incubated at 30 °C for 24–48 h.

Chromatographic runs were performed on an Acquity UPLC System (Waters, Milford, MA) coupled to a 3200 API Triple Quadrupole mass spectrometer (ABSciex, Foster City, CA) by a Turbo VTM interface equipped with a turbo ion spray probe used in positive ion mode. Data were recorded on an Acquity UPLC BEH C18 column (100 × 2.1 mm, i.d. 1.7 μm, Waters, Milford, MA). Water/ACN (90:10) mixture was used as eluent A and ACN (100 %) as eluent B. A linear gradient profile was

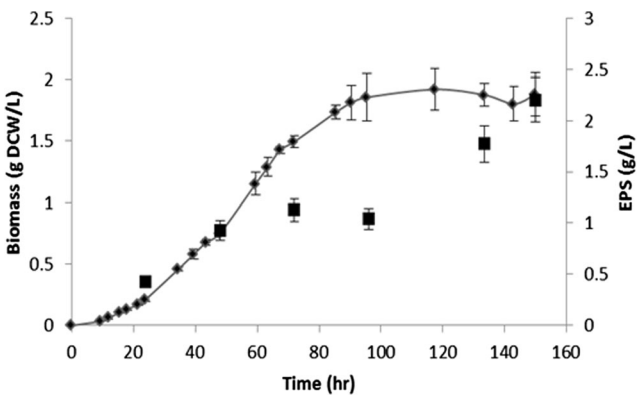

Fig. 1 Biomass (*black diamonds*) and exopolysaccharide (EPS; *black squares*) production profiles of *Halomonas smyrnensis* AAD6 cultures

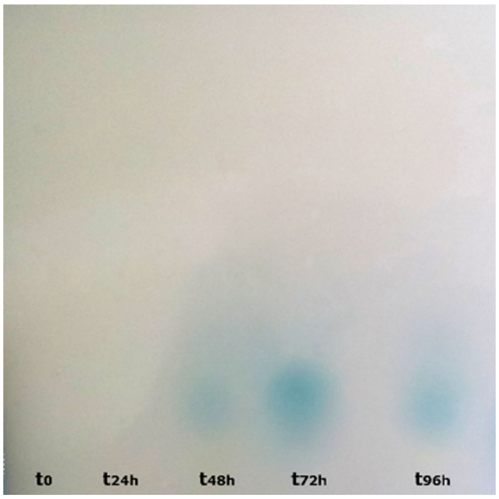

Fig. 2 Thin layer chromatography (TLC)-overlay assay performed on extracts of shaking cultures of *H. smyrnensis* AAD6 at different times (0, 24, 48, 72 and 96 h)

programmed from 100 % A to 100 % B in 1.0 min and kept constant over 3.0 min. Prior to the next injection, the column was equilibrated for 5 min. Separations were performed at a column temperature of 60 °C, using a flow rate of 0.8 mL/min and an injection volume of 2 μL.

Mass parameters were optimized by direct infusion of standard solutions into the ionization source. The instrument was tuned to improve the ionization and fragmentation process and to maximize sensitivity.

Multiple reaction monitoring (MRM) experiments were used to collect data, by setting the following source parameters: curtain gas (N_2): 20 psi, ion source gas (GS1): 55 psi, turbogas (GS2): 70 psi, desolvation temperature: 550 °C, collision activated dissociation gas (CAD): 4 a.u., and ion spray voltage: 5500 V. The ions monitored in Q1 included the parent AHL $[M+H]^+$, while in Q3 both $[M+H-101]^+$ and the lactone moiety at *m/z* 102 were monitored (Fig. 3). Tuning of AHL standards at 20 ng/mL allowed the optimization of the following sample parameters: declustering potential (DP): 27 V, entrance potential (EP): 10.6 V, collision energy (CE): 17 eV, cell exit potential (CXP): 3 V. The dwell time was set to achieve a total scan time of 0.10 s. The autosampler cooler was maintained at 27 °C. Analyst software (version 1.5.2; Applied Biosystems) was used for data registration.

All measurements were carried out in triplicate, and the results analyzed statistically using the Systat (Chicago, IL) version 7.0 software program to determine the average value and SEM of at least three experiments.

Samples for LC-MS analysis were taken at five different times of culture growth: $t=0$, $t=24$ h, $t=48$ h, $t=72$ h and $t=96$ h, and extracted as indicated above. The dried extracts were stored at −20 °C until analysis. For LC-MS runs each sample was reconstituted with 1 mL methanol.

The growth and EPS production profiles of *H. smyrnensis* AAD6 shaking cultures are reported in Fig. 1. Due to the high EPS concentration in the culture medium after 96 h, we selected previous times of growth for sampling at which to perform the extraction and to detect the signal production. The supernatant extracts were analyzed using the *A. tumefaciens* NTL4 (pCF218; pCF372) AHL bioreporter in the TLC-overlay assay and *H. smyrnensis* AAD6 culture supernatants were found to activate AHL bioreporter starting from $t=48$ h (Fig. 2). Identification of the AHL component responsible for the observed activity was assessed by ultra performance liquid chromatography (UPLC) electrospray ionization tandem mass spectrometry (ESI^+-MS/MS) using a triple quadrupole mass spectrometer. According to the UPLC method proposed by Fekete et al. 2007, a series of standards of AHLs, including acyl-, 3-oxo-, and 3-hydroxyl-derivatives with alkyl chain length from C-4 to C-18, were analyzed by monitoring MRM transitions of the molecular ion $[M+H]^+$ to the lactone moiety (*m/z* 102) and to the acyl chain ($[M-101+H]^+$ ions (Morin et al. 2003; Gould et al. 2006; Churchill et al. 2011).

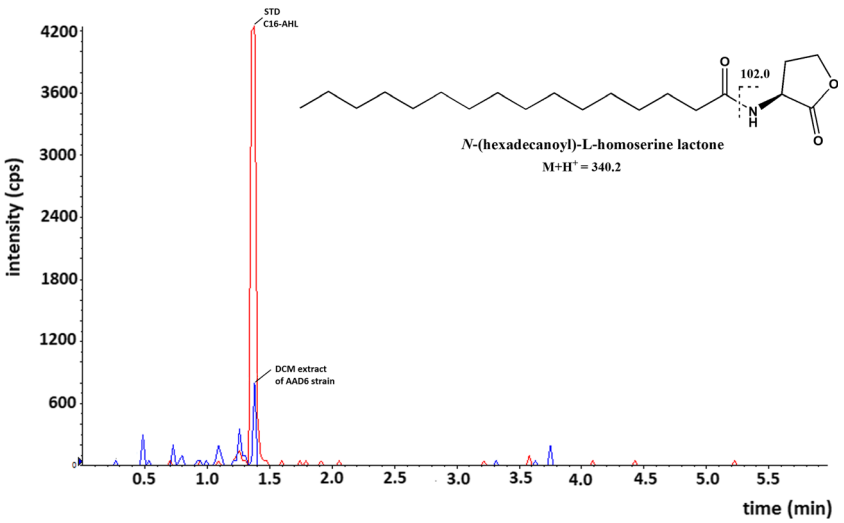

Fig. 3 Ultra performance liquid chromatography–multiple reaction monitoring (UPLC-MRM) analysis of C16-AHL standard (*red*) and dichloromethane (DCM) extract of AAD6 strain after 72 h growth (*blue*). The transition selected for MRM monitoring was *m/z* 340.2 > 102.0, which corresponds to the diagnostic fragmentation as indicated in the *inset* generating the homoserine lactone ion at *m/z* 102.0 from the molecular ion $M+H^+$ at *m/z* 340.2

The samples exhibiting a positive results in the TLC overlay-assay were analyzed by UPLC-mass spectrometry by MRM analysis under the same conditions used for AHL standards. A peak associated with the mass transition *m/z* 340.2 > 102.0 in the MRM analysis well matched with the C16-AHL standard (Fig. 3), thus strongly suggesting the occurrence of this metabolite in the bacterial extract.

The first evidence on the phenomenon of potential signal molecules of the homoserine lactone type in halophilic microorganisms was reported by Paggi et al. (2003). In that study, detection of QS signals in the haloalkaliphilic archaeon *Natronococcus occultus* and their role in the production of extracellular protease were described. Because the autoinducer activity was detected at the same stage (from exponential to stationary growth phase) at which protease activity was observed in the extracellular medium, the signal molecules could be involved in the production/activation of extracellular protease activity.

Later, Llamas et al. (2005) also reported AHL production by moderately halophilic bacteria, and, for the first time, also their chemical identification. They investigated QS activity in *Halomonas* species (*H. maura*, *H. eurihalina*, *H. ventosae* and *H. anticariensis*), describing a growth-phase dependent AHL production. A concomitant growth-phase dependent EPS production was also observed, thus the research group speculated that EPS synthesis could be controlled by AHL-based QS. Further analyses were performed on *H. anticariensis* FP35^T. Signal molecules were isolated and chemically characterized by means GC/MS and ESI MS/MS; four AHLs were identified: *N*-butanoyl homoserine lactone ©$_4$- AHL), *N*-hexanoyl homoserine lactone ©$_6$-AHL), *N*-octanoyl homoserine lactone ©$_8$-AHL) and *N*-dodecanoyl homoserine lactone ©$_{12}$-AHL). The hypothesis of a correlation between QS-activity and EPS production was confirmed by the observation of increased EPS levels in the culture of the analyzed strains supplemented with exogenous AHLs (Llamas et al. 2005).

According the data reported in literature, we investigated on the production of signal molecules in cell-free medium of *H. smyrnensis,* a halophilic and exopolysaccharide (EPS)-producing bacterium. Sugar analysis, methylation studies and NMR analysis of EPS revealed that the nature of this EPS was a levan with a repeating unit composed of b-(2,6)-D-fructofuranosyl residues. Maximum EPS concentration and yield was obtained at the pre-stationary phase of growth using a defined media containing sucrose as sole carbon source.

The EPS concentration and yield increased with biomass concentration, suggesting a growth-associated production, which was also in good agreement with the concomitant increase of net carbohydrate concentration with cell growth reported in a previous study (Poli et al. 2009). The growth-phase dependent EPS production by *H. smyrnensis*, which was reported previously, could lead to the hypothesis that EPS synthesis could be regulated by AHL-based QS, as well as described in the literature in other *Halomonas* spp.

In this paper, we report for the first time the isolation of a signal molecule, belonging to AHLs group, identified as *N*-Hexadecanoyl-L-homoserine lactone ©$_{16}$-AHL). Several bacterial species, including *Agrobacterium vitis*, *Paracoccus denitrificans*, *Rhodobacter capsulatus*, *Rhizobium leguminosarum*, and *Sinorhizobium meliloti* have been reported to produce AHLs with long acyl side chains (≥12 carbons) (Hao and Burr 2006; Llamas et al. 2004; Schaefer et al. 2002). To the best of our knowledge, there are no reports about production of AHLs with acyl chains longer than 12 carbons by halophilic bacteria. The growth-phase dependent EPS production by *H. smyrnensis* led us to hypothesize that EPS synthesis could be regulated by AHL-based QS. Further investigations will be aimed towards elucidation of the relationship between the isolated signal molecule ©$_{16}$-AHL) and EPS production.

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