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Detection of acylated homoserine lactones produced by *Vibrio* spp. and related species isolated from water and aquatic organisms

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Abstract

Aims: To assess the diversity in production of acylated homoserine lactones (AHLs) among *Vibrio* spp and related species.
Methods and Results: A total of 106 isolates, with representatives of 28 *Vibrio* spp and related species, were investigated for the production of AHLs. For this, a rapid method for the screening of AHLs was developed based on the use of bacterial biosensors using a double-layer microplate assay. At least one bacterial biosensor was activated in 20 species, *Agrobacterium tumefaciens* being the most frequently activated biosensor. One isolate of *Vibrio anguillarum*, *Vibrio rotiferianus* and *Vibrio metschnikovii* activated the *Chromobacterium violaceum* biosensor, which is not common among the Vibrionaceae family. For those species with more than one isolate, the biosensor activation profile was the same except for two species, *V. anguillarum* and *V. metschnikovii*, which varied among the different isolates.
Conclusions: AHL production was observed in the majority of the studied species, with a diverse biosensor activation profile.
Significance and Impact of the Study: The high diversity in AHL production is in consistence with the high diversity in ecological niches of the Vibrionaceae family. The absence of AHL detection in eight species warrants further work on their quorum-sensing systems.

Introduction

Vibrio spp. are Gram-negative, curved, rod-shaped, facultative anaerobe, gamma proteobacteria that can be found in aquatic environments as free-living forms or in symbiotic association with higher organisms such as marine algae and molluscs, corals, fish, shrimp, sponges or squids, some of which can also be pathogenic (Thompson *et al.* 2004). In this changing environment, bacteria are exposed to a variety of stimuli, but bacteria have evolved and adapt and survive through the regulation of certain genes in response to the stress. Some of these genes are regulated in a cell density-dependent manner, namely quorum sensing (Fuqua *et al.* 1994).

Cell-to-cell communication by quorum sensing is mediated by the production of one or more signalling molecules, also known as autoinducers, the concentration of which is related to the cell density of the producing cell and serves as a sensing mechanism of the population density to elicit a concerted response.

The quorum-sensing paradigm is the control of bioluminescence in *Vibrio fischeri*, a symbiotic micro-organism of the giant squid (*Euprymna scolopes*) through the LuxI-LuxR system, in which LuxI is the synthase of the acylated homoserine lactone (AHL) N-(3-oxo-hexanoyl)-L-homoserine lactone and LuxR a transcriptional activator responsible for the activation of the *lux* operon (Nealson *et al.* 1970; Engebrecht and Silverman 1984). Homologous to this system have been described in several other Gram-negative bacteria, although the AHLs produced by the LuxI homologues vary as well as the genes regulated by them (Whitehead *et al.* 2001).

AHLs have been the most studied quorum-sensing signalling molecules so far and seem to be very widespread among Gram-negative bacteria, although other signalling molecules have also been described, such as small peptides in Gram positive [see (Thoendel and Horswill 2010), for review] and the autoinducer 2, a furanosyl borate diester, used both by Gram-positive and Gram-negative bacteria (Miller and Bassler 2001; Federle and Bassler 2003). The AHLs are composed of a conserved homoserine lactone moiety and a fatty acid side chain that can vary in length (4–18 carbon atoms), the level of saturation and the oxidation degree of the side chain [see (Decho *et al.* 2011), for review].

Several quorum-sensing detection systems have been described, the majority of which make use of reporter strains that have different affinity to detect different molecules (Bassler *et al.* 1997; McClean *et al.* 1997; Cha *et al.* 1998). These strains have been genetically modified by placing reporter genes under the control of quorum-sensing-inducing promoters. Some of the strains detect a narrow range of AHLs such as *Pseudomonas putida* F117 (pAS-C8) that detects mostly C8 AHLs (Steidle *et al.* 2001), *Ps. putida* F117 (pKR-C12) that detects mostly 3-oxo-C12-HSL (Riedel *et al.*, 2001) or *Chromobacterium violaceum* CV26 that can detect short (C4-C8) mostly unsubstituted AHLs (McClean *et al.* 1997) or a broader AHL range such as *Agrobacterium tumefaciens* that can detect short to long AHLs (C4-C18) including most of the 3-oxo- and 3-hydroxilated AHL derivatives (Cha *et al.* 1998). The combination of different sensor strains, although cannot provide the exact AHL structure, is a simple method to have a first characterization of the AHL molecule, and the activation pattern of the different biosensors can reflect a different AHL production profile among different strains.

The quorum-sensing circuit of some *Vibrio* species are among the best characterized signalling circuits, particularly that of the aforementioned *V. fischeri*, as well as *Vibrio harveyi*, *Vibrio anguillarum* and *Vibrio cholerae* (Milton 2006). The aim of this study was to assess the production of AHLs among *Vibrio* spp isolated from water and organisms of different origin living in fisheries. This genus, comprising more than 60 species, a number that has noticeably increased in the last years, with their high genome diversity (Thompson *et al.* 2009) and their diverse ecological niches, is a good candidate to study the diversity of the quorum-sensing systems to better understand their interaction with the environment where they live.

Materials and methods

Bacterial strains, media and culture conditions

The bacterial strains used in the study are listed in Table 1. Cultures were grown at 30°C, agitated at 180 rev min^{-1} to an $OD_{600\ nm}$ of 0.2–0.4 in TSB medium for *Escherichia coli* MT102 (pSB403), *Ps. putida* F117 (pAS-C8 and pKR-C12) and *C. violaceum*, and AB medium for *Ag. tumefaciens* NTL4 (pZLR4) (Cha *et al.* 1998). When necessary, the concentration of the antibiotics used was as follows: tetracycline (20 $\mu g\ ml^{-1}$) for *E. coli*, gentamicin (30 $\mu g\ ml^{-1}$) for *Ag. tumefaciens* and *Ps. putida*, and kanamycin (50 $\mu g\ ml^{-1}$) for *C. violaceum*. The *Vibrio* strains were isolated from various organisms inhabiting fisheries (Austin *et al.* 1995, 1997; Kühn *et al.* 1996). All the *Vibrio* isolates were grown in modified TSB (TSB with 1.5%, TSB1.5 (w/v) NaCl or TSA1.5, when agar plates were used) (Difco, Barcelona, Spain) and incubated at 30°C unless otherwise stated.

Chemical reagents

Ethyl acetate (Panreac, Barcelona, Spain) was acidified with HCl (Sigma-Aldrich, Madrid, Spain) to a final concentration of 0.5% (v/v). *N*-hexanoyl-DL-homoserine lactone (C6-HSL), *N*-(β-ketocaproyl)-DL-homoserine lactone (oxo-C6-HSL), *N*-octanoyl-DL-homoserine lactone (C8-HSL) and *N*-dodecanoyl-DL-homoserine lactone (C12-HSL) were obtained from Sigma-Aldrich and were used to a final concentration of 0.1 mg ml^{-1}, unless otherwise stated. All the chemical reagents were of HPLC grade.

Biochemical identification

The biochemical characterization of the isolated strains was performed with the rapid miniaturized API20E strip (BioMérieux, La Balme, France) following the manu-

Table 1 Reporter strains used in the study, and AHLs detected with more affinity

Reporter strain	AHL detection range	Reporter gene	References
Escherichia coli MT102 (pSB403)	C4-C8-HSLs	*lux*	Winson *et al.* (1998)
Agrobacterium tumefaciens NTL4 (pZLR4)	3OH-HSLs and >C4-HSL	*β-gal*	Cha *et al.* (1998)
Chromobacterium violaceum CV26	C4-C6-HSLs	violacein production	McClean *et al.* (1997)
Pseudomonas putida F117 (pAS-C8)	C8-HSL	*gfp*	Steidle *et al.* (2001)
Ps. putida F117 (pKR-C12)	3-oxo-C12-HSL	*gfp*	Riedel *et al.* (2001)

AHLs, acylated homoserine lactones.

facturer's instructions, with minor modifications for the typing of *Vibrio* spp (Austin *et al.* 1997). However, as the API20E system focuses mostly on human pathogenic species, the IDENTAX® software (GNU Lesser General Public License; Free Software Foundation, Boston, MA) was used for identification purposes. This software has recently been updated with a biochemical key for the identification of *Vibrio* spp. (Flores *et al.* 2009). Those isolates showing a lower identification probability of 80% were confirmed by sequencing of the 16S rDNA, as described later. Those isolates belonging to the same species showing different biosensor activation profile were also confirmed by 16S rDNA sequencing.

16S rDNA sequencing

One millilitre of an overnight culture was centrifuged at 16 000 ***g*** for 5 min and resuspended in 100 μl of H_2O and boiled for 10 min and centrifuged at 16 000 ***g***. A volume of 2.5 μl of the supernatant was used as template for the PCR. Universal primers 27F and 1492R were used and the PCR performed as previously reported (Weisburg *et al.* 1991).

Nucleotide sequencing of the amplimer was performed in duplicate with an automated DNA sequencer (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany), model 377. Sequencing was carried out using the ABI PRISM Big Dye 3.1 Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Applied Biosystems, Madrid, Spain). The search for homologous DNA sequences was performed using the curated 16S rDNA database available at the Ribosomal Database Project web page (http://rdp.cme.msu.edu/index.jsp).

Phylogenetic analysis

Those isolates with the 16S rDNA matching more than one species with the same percentage of identity were phylogenetically analysed with the tools available at the Ribosomal Database Project web page (http://rdp.cme.msu.edu/index.jsp).

AHL detection by bacterial biosensors

A rapid screening method was developed to screen for the presence of AHLs in a 96-well microplate assay using different AHL bioreporter strains (Table 1). For this, 100 μl of TSA1.5 was added and allowed to dry. Next, 10 μl of an overnight culture of the *Vibrio* strain to test was spotted onto the agar surface and incubated for 2 h at 30°C, and a second layer containing the corresponding semisolid agar medium (0.7% agar) was inoculated with the sensor strain (3 : 2 v/v): TSB for *E. coli*, *Ps. putida* and *C. violaceum* sensor strains and AB containing X-Gal (80 μg ml^{-1}) for *Ag. tumefaciens*. Two negative controls were performed: inoculation of the biosensor strain alone and inoculation of the *Vibrio* strain alone to assess for the production of bioluminescence and/or the ability to grow in the selected agar. The microplates were incubated for 24 h at 30°C in a humid chamber. Activation of the reporter strains was considered a positive signal for the presence of AHLs and was determined as follows: emission of light for *E. coli* was measured with a Chemidoc™ XRS imaging system (Bio-Rad, Madrid, Spain), emission of green fluorescence for *Ps. putida* was measured by irradiation with a blue light lamp and visualized with gfp-filter glasses, the appearance of a violet pigment for *C. violaceum*, and the emergence of a blue spot for *Ag. tumefaciens* was determined at naked eye.

The performance of the microplate assay was confirmed with the cross-streak method previously reported (McClean *et al.* 1997), but this was only possible when the sensor strain was capable of growing in the medium used to grow the *Vibrio* strains, that is, only for *E. coli*, *Ps. putida* and *C. violaceum*.

Profiling of AHL production by thin-layer chromatography (TLC)

AHLs were extracted and analysed by TLC as described in (Garcia-Aljaro *et al.* 2008), with minor modifications. Briefly, 25 ml of free-cell overnight cultures of the relevant strains was mixed in the ratio of 1 : 1 with acidified ethyl acetate. The solvent phase was recovered with a pipette, and residual water in the extract was eliminated by adding anhydrous magnesium sulphate. The extract was then filtered through a Whatman paper 3 MM, dried with nitrogen flux air and resuspended in 50 μl of ethyl acetate. The presence of AHLs in the extracts was tested by C18 reverse-phase TLC (Uniplate RPS Reverse-Phase Hydrocarbon Impregnated Silica Gel; Analtech, Sigma-Aldrich, Barcelona, Spain) using methanol/water (60 : 40, v/v) (Cha *et al.* 1998). The plates were dried and overlaid with 50 ml of soft agar containing the biosensor strain and incubated as described previously.

Results

A total of 106 *Vibrio* spp. isolates of different origins including water and aquatic organisms inhabiting fisheries were identified by biochemical fingerprinting using IDENTAX® software, and sequencing of the 16S rDNA, in case of ambiguity. The isolates belonged to 28 different species, which are shown in Table 2. *V. anguillarum* was the species with a higher number of isolates (38 isolates), followed by *Vibrio vulnificus* (nine isolates), *V. harveyi* (seven isolates) and *Vibrio alginolyticus*, *Photobacterium damselae*, *Vibrio mediterrani*, *Vibrio metschnikovii*, all of them with

Table 2 Activation of the different sensor strains with the microplate assay: EC, *Escherichia coli* MT102 (pSB403); AT, *Agrobacterium tumefaciens* NTL4 (pZLR4); PpC8, *Pseudomonas putida* F117 (pAS-C8); PpC12, *Ps. putida* F117 (pKR-C12); *n*, number of isolates

	n	EC	AT	PpC8	PpC12	CV	Origin
Aliivibrio logei	1		+				Water
Aliivibrio salmonicida	1	+	+	+	+		*Oncorhynchus nerka*
Aliivibrio wodanis	1						*Penaeus orientalis*
Vibrio anguillarum	1	+	+	+	+	+	*Sparus aurata*
V. anguillarum	37	+	+	+	+		*Dicentrarchus labrax*, *Scophthalmus maximus*, *Water*, *Salmo salar*, *Plecoglossus altivelis*, *Pagrus major*, *Anguilla* sp., *Seriola lalandi*, *Chanos chanos*, *Oncorhynchus nerka*, *Oncorhynchus mykiss*, *Seriola lalandi*
Photobacterium damselae	5						*Penaus monodon*, *Water*
Photobacterium leiognathi	1		+				*Chanos chanos*
Photobacterium ganghwense	1	+	+	+			*Penaeus* sp.
Salinivibrio costicola	2						Water
Vibrio aestuarianus	1		+				*Anguilla* sp.
Vibrio alginolyticus	5						Penaeus monodon, water
Vibrio chagasii	2		+				*Abramis brama*
Vibrio cholerae	1		+				*Acanthurus coeruleus*
Vibrio diazotrophicus	2		+				*Artemia* sp.
Vibrio fluvialis	3	+	+	+	+		*Oncorhynchus kisutch*
Vibrio harveyi	7		+				*Acanthurus coeruleus*, *Penaeus monodon*, water
Vibrio ichthyoenteri	2		+				Water
Vibrio lentus	1		+				*Crassostrea* sp.
Vibrio litoralis	1						*Sparus aurata*
Vibrio mediterranei	5	+	+	+	+		*Abramis brama*, *Mytilus edulis*
Vibrio metschnikovii	1	+	+	+	+	+	*Penaeus monodon*
V. metschnikovii	4	+	+	+	+		Water
Vibrio mimicus	1		+				*Anguilla* sp.
Vibrio mytili	2						*M. edulis*
Vibrio natriegens	1						*Crassostrea* sp.
Vibrio parahaemolyticus	3						*P. orientalis*
Vibrio proteolyticus	2		+	+			*Mercenaria mercenaria*
Vibrio rotiferianus	1	+	+	+		+	*Paguara* sp.
Vibrio splendidus	2		+	+			*Abramis brama*, *Scophthalmus maximus*
Vibrio vulnificus	9		+				Water, *Anguilla* sp., *Penaeus monodon*

five isolates. For the remaining species, the number of isolates varied among 1 and 4 (Table 2). As shown in Table 2, the origin of the isolates even for those belonging to the same species was highly diverse, including water samples, different kind of fish, molluscs, crustaceae and rotifers. All these strains were part of our personal culture collection of *Vibrio* spp. isolates generated from different studies (Austin *et al.* 1995, 1997; Kühn *et al.* 1996).

Taking into consideration the high diversity of the isolates concerning the origin and the high number of different species, we were interested in the diversity of the AHL produced by the isolates. For this, a rapid screening method for the detection of AHLs was developed using a 96-well microplate double-layer diffusion assay. The assay was simple, and consisted in depositing a first layer of TSA1.5 agar suitable for the growth of the *Vibrio* isolates at the bottom of the microplate, followed by inoculation of the test strain and overlaying it with a second layer of semisolid agar in which the sensor strain to test was able to grow. With this method, all the isolates were screened for the production AHLs (Fig. 1). The majority of the isolates (85%) corresponding to 20 of 28 species analysed in the study produced AHL capable of inducing at least one of the sensor strains. The bioreporter strains were activated by the different species as follows: *Ag. tumefaciens* (20), *Ps. putida* (pAS-C8) (9), *E. coli* (7), *Ps. putida* (pKR-C12) (5) and *C. violaceum* (3). Those strains that did not activate any of the sensors were double-checked with the previously described 'cross-streak' method in which the sensor strain is streaked perpendicular to the test strain. The results were confirmed for the sensor strains *E. coli*, *C. violaceum*, *Ps. putida* C8 and C12. However, because no medium was able to support the simultaneous growth of *Vibrio* spp. and related species and *Ag. tumefaciens*, confirmation of the absence of *Ag. tumefaciens* inducing AHLs was performed by extraction of AHLs and analysis by TLC as discussed later (Fig. 2). It has to be noted that in the case of one *V. harveyi* isolate,

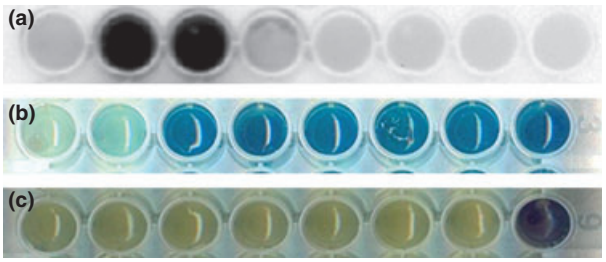

Figure 1 Detection of AHLs with the microplate assay method. (a) Negative image of AHL-activated *Escherichia coli* biosensor (black wells); (b) AHL-activated *β*-galactosidase in the *Agrobacterium tumefaciens* biosensor (blue wells); (c) activation of violacein production in the *Chromobacterium violaceum* biosensor by an AHL-producing strain (violet well).

the luminescence of the bacterium itself interfered with the microplate assay method with the *E. coli* luminescent bioreporter strain, and the cross-streak and/or the TLC method was necessary to confirm the production of AHLs. For this reason and to detect bacterial luminescence, a negative control in which each strain was inoculated alone was performed. AHLs were not detected in a total of eight species with the bioreporter strains used in the study including *Aliivibrio wodanis* (1), *Vibrio parahaemolyticus* (3), *Salinivibrio costicola* (2), *Vibrio natriegens* (1), *Vibrio mytili* (2), *Vibrio litoralis* (1), *P. damselae* (5) (previously *V. damselae*) and *V. alginolyticus* (5). In summary, seven different sensor activation profiles were detected among the different species (Table 2). It has to be noted that for those species with more than one isolate, the biosensor activation profile was the same except for two species, *V. anguillarum* and *V. metschnikovii*, which varied among the different isolates (Table 2).

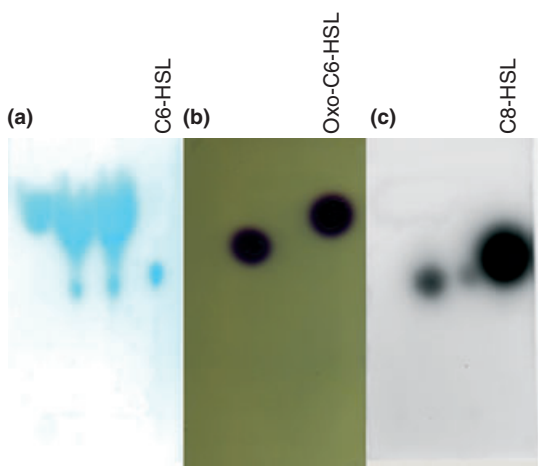

Figure 2 Example of AHLs detection with the thin-layer chromatography method: (a) *Agrobacterium tumefaciens*; (b) *Chromobacterium violaceum*; (c) *Escherichia coli*.

In view of the diversity in the activation of the different bioreporter strains even at the species level, at least one isolate showing a different activation profile was analysed by TLC to visualize the number and estimate the type of AHLs produced, using commercial AHL standards as reference molecules. The number of molecules detected in the chromatography was more than one for the majority of isolates when analysed with the *Ag. tumefaciens* strain. In the case of the species activating all the bioreporter strains but *C. violaceum*, they were shown to produce between three and five AHLs by TLC, and those activating the *C. violaceum* strain possibly carried a C6-HSL according to the R_f value obtained. It has to be noted that in any case the TLC analyses confirmed the results of the microplate assay.

Discussion

Numerous quorum-sensing detection systems have been developed for the detection of AHLs, some of which make use of bioreporter strains. In this study, we investigated the production of AHLs in members of the Vibrionaceae family with a rapid method based on the use of bioreporter strains in a double-layer assay using 96-well microplate. The developed microplate assay method was consistent with the results obtained by the other previously described methods such as the 'cross-streak' (McClean *et al.* 1997) and/or TLC (Shaw *et al.* 1997) in terms of activation of the sensor strains. Moreover, it has the advantage that can overcome the limitation of the 'cross-streak' method in that each couple sensor-test strain can be inoculated in its optimal medium without interfering to each other.

All the species showing AHL production activated the *Ag. tumefaciens* sensor strain, which is the one that is activated by a broad range of AHLs, and the only one that can be activated by hydroxylated AHLs (Cha *et al.* 1998). In fact, activation of only the *Ag. tumefaciens* bioreporter strain was the most frequently detected profile, suggesting that these species are able to produce hydroxylated AHLs, which is the case for instance in *Vibrio scophthalmi* (Garcia-Aljaro *et al.* 2008). The second profile most frequently detected was *E. coli*, *Ag. tumefaciens*, *Ps. putida* C8 and *Ps. putida* C12 represented by five species. Surprisingly, one strain of the following species activated the *C. violaceum* sensor, *V. anguillarum*, *Vibrio rotiferianus* and *V. metschnikovii*; which is not a common feature among the *Vibrionaceae* family. This sensor strain is activated by medium-sized AHLs (C4-C8), mostly with no substitutions. It has to be noted that a certain degree of diversity in the activation of the sensor strains was observed at an interspecies as well as at an intraspecies level. For instance, two different profiles were observed for *V. anguillarum* and *V. metschnikovii* species. However,

in the case of the eight species that were not capable of activating any sensor strain, it demonstrates that they are not producing the most frequent AHLs produced by the majority of species of the Vibrionaceae family, or at least under the conditions tested. It represents almost a 30% of the species analysed in the study belonging to different genera. Therefore, the existence of novel AHLs cannot be excluded. Further work will be performed to study the presence of AHL synthases in these species.

In the TLC assay using the *Ag. tumefaciens* bioreporter strain, the sensor was activated by more than one molecule in the majority of the strains. However, some authors have reported that this biosensor detects AHLs with a certain degree of unspecificity, because in some cases it was not possible to isolate and characterize the AHL inducing the sensor strain (Garcia-Aljaro *et al.* 2008). However, because we used other biosensors with different AHL affinity, at least those molecules with a similar retention factor (R_f) on the TLC assays with the different biosensors should be considered as AHLs.

In a recent study, a total of 25 strains belonging to the Vibrionaceae family were screened for the production of AHLs by three biosensors, one of which was the *C. violaceum* sensor used in our study (Yang *et al.* 2011). A total of 11 species produced AHLs as detected by the *Ag. tumefaciens* KYC55 sensor strain (Han *et al.* 2010), which is theoretically more sensitive than our *Ag. tumefaciens* NTL4 strain, but none of them activated the *C. violaceum* sensor strain. Our results are in accordance with those of Yang in the fact that three of these species, *Al. costicola*, *P. damselae* and *V. natriegens*, did not produce AHLs or at least not in sufficient amount for being detected by *Ag. tumefaciens*. However, our *Vibrio splendidus* and *V. metschnikovii* isolates produced AHLs detected in some strains by *E. coli*, *Ag. tumefaciens*, *Ps. putida* (pAS-C8), *Ps. putida* (pKR-C12) and even *C. violaceum* CV26 in the case of a *V. metschnikovii* and a *V. rotiferianus isolate*. Concerning the number of different AHLs produced, significant differences among the most well-studied vibrios have been reported. For instance, *V. fischeri* has been reported to produce 3-oxo-C6-HSL and C6-HSL (McDougald *et al.* 2000); *V. salmonicida*, 3-oxo-C6-HSL and C6-HSL (Bruhn *et al.* 2005); *V. anguillarum* C6-HSL, 3-hydroxy-C6-HSL, 3-oxo-C10-HSL and in minor amounts, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL and 3-oxo-C12-HSL (Buchholtz *et al.* 2006); and *V. vulnificus*, C4-HSL in strains isolated from fish but not in strains isolated from septicaemic cases (Valiente *et al.* 2009). In the study of (Yang *et al.* 2011), although the AHLs were not chemically characterized, they were tentatively identified by comparing the R_f values of AHL standards. In this way, it was shown that *Vibrio aestuarianus* putatively produced 3-oxo-C8 and C8-HSL; *V. mediterranei* and *Aliivibrio logei*, produced 3-oxo-C6-HSL and three other AHL-sensor activating molecules not characterized; *Vibrio fluvialis*, C6-HSL and C8-HSL and *Vibrio proteolyticus*, one nonidentified AHL-like molecule capable of activating the *Ag. tumefaciens* biosensor.

The results presented in our work enlarge the list of AHL-producing species members of the Vibrionaceae family. The different AHLs could be produced by a single synthase or by different synthases acting in different quorum-sensing systems as reported for *V. anguillarum* LuxI and LuxM synthases (Milton 2006). In conclusion, there is a high diversity of AHLs production among different species but also a certain level of intraspecific diversity among members of the same species which is in consistence with the high diversity of the Vibrionaceae family. The ecological significance of these differences remains unknown and warrants further studies.

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