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Article

# Characterization of *N*-Acyl Homoserine Lactones in *Vibrio tasmaniensis* LGP32 by a Biosensor-Based UHPLC-HRMS/MS Method

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**Abstract:** Since the discovery of quorum sensing (QS) in the 1970s, many studies have demonstrated that *Vibrio* species coordinate activities such as biofilm formation, virulence, pathogenesis, and bioluminescence, through a large group of molecules called *N*-acyl homoserine lactones (AHLs). However, despite the extensive knowledge on the involved molecules and the biological processes controlled by QS in a few selected *Vibrio* strains, less is known about the overall diversity of AHLs produced by a broader range of environmental strains. To investigate the prevalence of QS capability of *Vibrio* environmental strains we analyzed 87 *Vibrio* spp. strains from the Banyuls Bacterial Culture Collection (WDCM911) for their ability to produce AHLs. This screening was based on three biosensors, which cover a large spectrum of AHLs, and revealed that only 9% of the screened isolates produced AHLs in the defined experimental conditions. Among these AHL-producing strains, *Vibrio tasmaniensis* LGP32 is a well-known pathogen of bivalves. We further analyzed the diversity of AHLs produced by this strain using a sensitive bioguided UHPLC-HRMS/MS approach (Ultra-High-Performance Liquid Chromatography followed by High-Resolution tandem Mass Spectrometry) and we identified C10-HSL, OH-C12-HSL, oxo-C12-HSL and C14:1-HSL as QS molecules. This is the first report that documents the production of AHL by *Vibrio tasmaniensis* LGP32.

**Keywords:** quorum sensing; *N*-acyl-homoserine lactone (AHL); *Vibrio tasmaniensis* LGP32; fractionation; biosensors; UHPLC-HRMS/MS

## 1. Introduction

Bacteria of the genus *Vibrio* are ubiquitous marine bacteria belonging to the *Gammaproteobacteria* class and this genus includes both non-pathogenic and pathogenic species. Among the 133 described species of *Vibrio* ([www.bacterio.net](http://www.bacterio.net)), at least 12 (e.g., *V. cholerae*, *V. vulnificus*, *V. parahaemolyticus*) are well known to be highly pathogenic to humans and a large number of other strains are pathogens of a wide range of marine organisms [1–3]. These opportunistic bacteria are also able to colonize diverse substrates, to participate in biofilm production [4,5], and to associate with phytoplankton, zooplankton, marine vertebrates and invertebrates [6–9]. Quorum sensing (QS) has been shown to be involved in many of these processes and associations [10,11], whereby the population density regulates gene expression, through the emission of self-generated small signal molecules named autoinducers (AI), to obtain a concerted physiological response [12–14].

Type 1 autoinducers (AI-1) also called *N*-acyl homoserine lactones (AHLs) are signaling compounds used by many bacteria for communication among single or closely related species [15–18]. In Gram-negative bacteria, the AHLs are synthesized by one or more synthases whose LuxI and LuxM/AinS are the most widespread [19–21]. Since the first investigations on QS in the symbiotic and bioluminescent *V. fischeri*, other *Vibrio* species were shown to communicate using AHLs [22–24]. QS molecules and signaling pathways are well known for *V. cholerae*, *V. fischeri*, *V. harveyi*, *V. vulnificus* and *V. anguillarum* as are their roles in bioluminescence, pathogenicity and biofilm formation [25]. Nonetheless, the prevalence and diversity of AHL-based QS in other environmental strains of *Vibrio* remains poorly understood.

Direct in situ studies of AHLs production in the marine environment have been limited and hampered by the fact that AHLs are likely released locally in microenvironments of high cellular concentration and thus at low total concentrations in a water sample. Therefore, quantifying AHL production directly in the environment is extremely difficult [26]. To overcome this problem, the isolation and identification of environmental strains able to produce AHLs and the characterization of these compounds appear as the best alternative. For these purpose, several detection approaches have been previously developed and most of them are using bioreporters able to detect a wide range of AHLs [27–29]. Most of these bioreporters are genetically modified strains, where reporter genes such as violacein or phenazine production,  $\beta$ -galactosidase or green fluorescent protein (GFP), are under the control of AHL-based-quorum sensing inducing promoters [29,30]. The use of multiple reporter strains does not allow the characterization of individual AHL molecules but provides an activation pattern reflecting a specific AHLs production phenotype in the defined culture condition [22,31]. Thus, in the past 10 years, numerous studies characterized AHLs—in combination or not with reporters—using analytical chemistry tools. The characterization of known AHLs is most commonly achieved by thin-layer chromatography (TLC) or High Performance Liquid Chromatography (HPLC) and comparison to standards [31–34]. However, since the diversity of AHLs is far from completely described [35], the characterization of *novel* AHLs requires the combination of chromatography with structural analysis, like Gas Chromatography coupled with Mass Spectrometry (GC-MS; [36]), HPLC coupled with tandem Mass Spectrometry (HPLC-MS/MS) or Nuclear Magnetic Resonance (NMR) [37]. The recent development of more sensitive and accurate techniques such as Ultra-High Performance Liquid Chromatography (UHPLC) combined to High Resolution Mass Spectrometry (HRMS) have yielded a high diversity of *novel* AHL molecules [38,39].

*Vibrio tasmaniensis* strain LGP32, previously named *Vibrio splendidus*, has been widely used as a model organism for the study of host-pathogens relationship in bivalves [40–42]. This strain had been isolated from diseased oysters during mortality events in France and is a facultative intracellular pathogen that attach and invade oyster hemocytes [43]. The complete genome analysis of LGP32 has revealed that—like other pathogenic ones, such as *V. harveyi*—this strain, harbors QS systems based on three different autoinducers (AHL, CAI-1 and AI-2) [16]. Interestingly, De Decker et al. showed in 2013 a possible involvement of QS in the virulence mechanisms of LGP32 [44], but remarkably the AHL molecules associated with this strain have not yet been characterized.

The aim of this study was to first evaluate the AHL production among environmental *Vibrio* spp. strains isolated from a large diversity of marine environments. The result of this screening led us to focus on the chemical diversity of AHLs in *Vibrio tasmaniensis* LGP32 using an optimized protocol to potentially maximize the discovery of *novel* AHLs. This protocol includes the use of large extraction volumes (3 L) followed by a biosensor-based screening of HPLC fractions prior to structural characterization by UHPLC-HRMS/MS. In this study, we also assessed the limit of detection of various approaches and extended the range of tested AHLs compared to previous studies. Our methodological approach allowed, for the first time, the characterization of AHL compounds involved in the QS of the important bivalve pathogen *Vibrio tasmaniensis* LGP32.

## 2. Materials and Methods

### 2.1. Culture Collection and Strains Identification

The Banyuls Bacterial Culture Collection (BBCC) is referenced in the World Data Center for Microorganisms as WDCM911 and harbors more than 2000 bacterial strains isolated from different geographical sites which are mostly heterotrophic marine bacteria identified on the 16S rRNA gene sequence [45]. Genomic DNA of each of the strains was extracted with the Wizard Genomic DNA purification kit (Promega, Charbonnières-les-Bains, France) as previously described [46]. PCR was performed using the universal primers targeting bacteria, 27Fmod (AGRGTTCGATC-MTGGCTCAG) [47] and 1492Rmod (TACGGYTACCTTGTTAYGACTT) [48]. PCR products were purified using the Agencourt AMPureXp purification kit (Beckman Coulter, Villepinte, France) and sequenced as described previously with an AB3130xl genetic analyzer (Applied Biosystems, Courtaboeuf, France). All molecular biology instrumentation was available through the Bio2Mar platform at the Observatoire Oceanologique de Banyuls-sur-Mer. The 16S rRNA gene sequences were compared to sequences within the NCBI nt database using the Basic Local Alignment Search Tool—2 sequences [49,50]. For the QS screening we selected all strains in the BBCC Culture Collection with a similarity percent above 98% to known *Vibrio* species (i.e., Table 1). The List of the 87 tested strains with their origin, their identification by 16S rRNA gene sequence and their GenBank accession numbers can be found in Table S1 of the Supplementary Material.

**Table 1.** Activation patterns of *Vibrio* species when tested against three biosensor strains: F117, *Pseudomonas putida* (pKR-C12); MT102, *Escherichia coli* (pJBA-132) and CV026, *Chromobacterium violaceum*.

Closest Relative Species	Number of Isolates	BBCC Code	CV026	MT102	F117
<i>Vibrio atlanticus</i>	1	2313	-	-	-
<i>Vibrio brasiliensis</i>	2	493, 494	-	-	-
<i>Vibrio breoganii</i>	1	1958	-	-	-
<i>Vibrio campbellii</i>	3	62, 416, 2415	-	-	-
<i>Vibrio chagasii</i>	4	583, 586, 640, 2353	-	-	-
<i>Vibrio cortegadensis</i>	2	529, 1974	-	-	-
<i>Vibrio gallaecicus</i>	4	528, 2315, 2319, 2327	-	-	-
<i>Vibrio gigantis</i>	16	503, 530, 853, 1230, 1232, 1233, 1972, 1973, 1980, 1982, 1989, 2045, 2312, 2357, 2372, 2412	-	-	-
<i>Vibrio harveyi</i>	7	558, 576, 579, 605, 615, 626, 2366	-	-	-
<i>Vibrio hemi-centroti</i>	2	2269, 2311	-	-	-
<i>Vibrio ichthyoenteri</i>	2	490, 491	-	-	-
<i>Vibrio lentus</i>	3	495, 850, 851	-	-	-
<i>Vibrio maritimus</i>	1	2338	-	-	-
<i>Vibrio metschnikovii</i>	2	1026, 1055	-	+	+
<i>Vibrio mytili</i>	1	2428	+	-	-
<i>Vibrio natriegens</i>	1	546	-	-	-
<i>Vibrio neptunius</i>	1	496	-	-	-
<i>Vibrio ordalii</i>	1	1015	+	+	+
<i>Vibrio owensii</i>	3	1143, 1169, 1955	-	-	-
<i>Vibrio pectenicida</i>	1	1971	-	-	-
<i>Vibrio pomeroyi</i>	2	502, 1962	-	-	-
<i>Vibrio rumoiensis</i>	2	1210, 1211	-	-	-
<i>Vibrio scophtalmi</i>	3	1228, 1237, 1238	-	+	+
<i>Vibrio scophtalmi</i>	4	2361, 2365, 2370, 2413	-	-	-
<i>Vibrio shilonii</i>	3	2339, 2351, 2363	-	-	-
<i>Vibrio sinaloensis</i>	1	2347	-	-	-
<i>Vibrio splendidus</i>	8	66, 67, 165, 239, 498, 500, 527, 852	-	-	-
<i>Vibrio tasmaniensis</i>	1	526	-	-	-
<i>Vibrio tasmaniensis</i> LGP32	1	2197	+	+	-
<i>Vibrio tubiashi</i>	4	620, 1231, 2159, 2190	-	-	-

## 2.2. Biosensor Assays

The detection of AHLs in culture supernatants and HPLC fractions (see details in Section 2.3) followed previously described protocols using the biosensors *Pseudomonas putida* (*P. putida*), *Escherichia coli* (*E. coli*) and *Chromobacterium violaceum* (*C. violaceum*). The biosensors *Pseudomonas putida* F117 (pRK-C12; Kmr; *ppuI::npt*) and *Escherichia coli* MT102 (pJBA132) were used for the detection of AHLs in liquid medium and *Chromobacterium violaceum* CV026 was used for the detection of AHLs in solid medium [51–53]. Briefly, 50  $\mu\text{L}$  of culture supernatants, obtained by centrifugation of 2 mL of culture in Marine Broth (MB), grown overnight at 25 °C under shaking (100 RPM), and 20  $\mu\text{L}$  of HPLC fractions at 10  $\text{mg}\cdot\text{mL}^{-1}$  in dimethyl sulfoxide (DMSO) diluted in Luria Bertani (LB) broth (1/4 *v:v*), were tested in triplicate. For the biosensors *E. coli* and *P. putida*, microplates were incubated respectively at 30 °C and 37 °C and OD was measured at 535 nm after 0, 5 and 24 h of incubation. OD620 was also measured to control for biosensor cell growth [30,53–55]. For the biosensor *C. violaceum*, culture plates were incubated at 30 °C and purple zones of violacein production were inspected after 24 h [51]. For all tests, negative controls consisted of biosensor cultures without supernatant or fractions, and sterile LB medium. Biosensor cultures with addition of commercial AHLs (C6-HSL and oxo-C10-HSL, Cayman Chemical, Ann Arbor, MI, USA) were used as positive controls. To determine the AHL concentration range of detection of each biosensor, a dilution range from 0.01 to 25,000 nM of 27 commercial AHL standards (Cayman Chemical, see Table 2) was tested following the protocols described above.

**Table 2.** Limit of detection ( $\text{nmol}\cdot\text{L}^{-1}$ ) of AHL standards using UHPLC-HRMS and three different biosensors: F117, *Pseudomonas putida* (pKR-C12); MT102, *Escherichia coli* (pJBA-132) and CV026, *Chromobacterium violaceum*. ND: not detected. MD: missing data.

	Limit of Detection ( $\text{nmol}\cdot\text{L}^{-1}$ )			
	CV026	MT102	F117	UHPLC-HRMS
C4-HSL	250	ND	ND	>500
C6-HSL	2.5	0.631	312.38	3.64
OXO-C6-HSL	10	<0.001	ND	10.90
C7-HSL	1	0.094	ND	5.33
C8-HSL	5	1.125	212.49	6.50
OXO-C8-HSL	10	0.0024	0.76	6.15
OH-C8-HSL	100	ND	7.077	MD
C9-HSL	5	1.93	2.89	7.37
C10-HSL	100	74.52	1.5	4.56
OXO-C10-HSL	1000	0.07	<0.001	2.91
OH-C10-HSL	2.5	ND	13.43	3.23
C11-HSL	ND	ND	<0.001	9.11
C12-HSL	ND	ND	0.01	5.07
OXO-C12-HSL	ND	0.702	<0.001	21.28
OH-C12-HSL	ND	ND	0.125	2.39
C13-HSL	ND	ND	0.00475	14.78
C14-HSL	ND	ND	0.608	11.82
C14:1-HSL	ND	0.366	0.00535	9.41
OXO-C14-HSL	ND	0.76	<0.001	6.79
OXO-C14:1-HSL	ND	ND	0.0606	4.71
OH-C14-HSL	ND	ND	0.492	36.58
C15-HSL	ND	ND	0.094	15.11
C16-HSL	ND	ND	12.01	16.30
C16:1-HSL	ND	0.1	0.023	14.68
OXO-C16:1-HSL	ND	ND	6.28	6.75
C18-HSL	ND	ND	ND	10.48
C18:1-HSL	ND	ND	7.8	28.56

### 2.3. AHL Extraction and HPLC Fractionation of LGP32

LGP32 was cultured in 3 L of Marine Broth (Difco, Le pont de Claix, France) at 25 °C under shaking (100 RPM) for 24 h (representing a late exponential phase, pH 7.5). A liquid-liquid extraction of the culture was performed with ethyl acetate (1/3 *v:v*) in a separatory funnel. The organic phase was evaporated to dryness and the extract was re-suspended in 1 mL of HPLC grade DMSO. The extract was fractionated using a separative HPLC system with two Varian Prep Star pumps, a manual injector, a Dionex Ultimate 3000 RS variable wavelength detector and a Dionex Ultimate 3000 fraction collector (Thermo Scientific, Courtaboeuf, France). The column was a Phenomenex Luna C18 (21.2 mm × 250 mm), with 5 µm particle size, and the flow rate was set to 20 mL·min<sup>-1</sup>. The mobile phase consisted of HPLC grade H<sub>2</sub>O and CH<sub>3</sub>CN at different proportions starting at 70:30 for 3 min, followed by a 12 min linear gradient from 70:30 to 0:100, followed by 100% CH<sub>3</sub>CN for 10 min. 22 fractions were collected every minute between 3 and 25 min. The solvent was removed with a HT-4X system (Genevac, Biopharma Technologies France, Lyon, France), each fraction was dissolved in 100 µL DMSO and diluted at 1/4 with LB medium (*v/v*) to perform the biosensor tests. Positive fractions were further analyzed by UHPLC-HRMS/MS. pH was controlled at each step of our experimental process and maintained in between 6 and 7.

### 2.4. AHL Detection by UHPLC-HRMS/MS

Prior to injection, fractions were diluted at 1 mg·mL<sup>-1</sup> and 5 µL were injected. UHPLC-MS analyses were performed with a Dionex Ultimate 3000 UHPLC-HESI HRMS Q-Exactive focus system (Thermo Scientific) controlled by the Xcalibur software. The column was a Hypersil GOLD C18 (2.1 mm × 150 mm) with 1.9 µm particle size (Thermo Scientific). The column oven was set to 50 °C. The flow rate was maintained at 0.8 mL·min<sup>-1</sup>. The mobile phase was composed of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (B). A gradient profile was used, starting with 100% of A, and keeping this composition constant for 5 min. The proportion of B was linearly increased to 100% in 5 min, and was left at 100% for 5 min. Settings for the ion source were: 20 aux gas flow rate, 75 sheath gas flow rate, 4 µA spray current, 3 kV spray voltage, 350 °C capillary temperature, 450 °C heater temperature, and 40 S-lens RF and nitrogen was used as nebulizing gas by the ion trap source.

Firstly, MS and MS/MS profiles were recorded alternating between a full scan (scan range 130 to 900 *m/z*) and All Ion Fragmentation (AIF) mode [scan range 60 to 600 *m/z*, normalized-collision energy (NCE) 25] to determine molecular weights and identify chromatographic peaks generating fragment ions at *m/z* 102.0555. Mass resolution was set at 35,000, AGC target was 1 × 10<sup>6</sup> and 5 × 10<sup>4</sup> respectively for the full scan and AIF mode, and injection time was 40 ms. The study of mass spectra obtained for our standard molecules and the study of Patel et al. [35] reveal that fragment ions at *m/z* 102.055, 84.045, 74.06 and 56.05 are specific of the homoserine lactone (HSL) moiety and these signals were chosen as the specific ions indicating the presence of AHL-type compounds. In a second step, we used a SIM (resolution 35,000) and dd-ms2 (resolution 17,500) mode to confirm the AHLs identification. MS/MS scans were isolated using an isolation width of 3.0 Da, fragmentations were performed at 17,500 with a collision energy of 20 eV. The limit of detection (LOD) of our UHPLC-HRMS method was established based on the standard deviation of the response to the 27 AHLs at 50 nmol·L<sup>-1</sup> and the slope of a 10-fold concentration range (*R*<sup>2</sup> values > 0.99). Briefly, a calibration curve was constructed using a simple linear regression analysis from the injection of AHL standard solution mixtures at concentration ranging from 5 to 500 nmol·L<sup>-1</sup>. Each AHL was injected 10 times at 50 nmol·L<sup>-1</sup> and LOD was expressed as 3.3 times the standard deviation (LOD = 3.3SD).

## 3. Results

### 3.1. Biosensors Strains & UHPLC-HRMS/MS AHL Detection Limits

To define the specificity and the sensitivity of the three biosensors strains, we evaluated their responses to 27 commercially available AHL molecules (Table 2). The *C. violaceum* biosensor (CV026)



showed a highly specific response to short chain AHLs (<10 carbons in the acyl chain) with a high sensitivity for C6-HSL (2.5 nmol·L<sup>-1</sup>), C7-HSL (1 nmol·L<sup>-1</sup>) and OH-C10-HSL (2.5 nmol·L<sup>-1</sup>). The biosensors *E. coli* MT102 detected mostly short acyl side chain AHLs and *P. putida* F117 showed the lowest specificity, by detecting 23 of 27 AHLs, while exhibited highest sensitivity to 3-oxo-HSL (LOD < 0.001 nmol·L<sup>-1</sup>; Table 2). Overall, GFP-based biosensors, MT102 and F117, showed the highest sensitivity levels to the most tested molecules. Finally, C4-HSL was only detected by *C. violaceum*.

Considering the overlap between the biosensors CV026 and MT102 and the suitability of GFP-based biosensors for high-throughput analyses, our fractionation and structural determination by UHPLC-HRMS/MS was solely based on detection by *E. coli* MT102 and *P. putida* F117 as biosensors. We evaluated the LOD of our UHPLC/HRMS-MS devices for 27 commercially available AHLs also used as AHL standards in our study and these detection limits were between 2.39 nmol·L<sup>-1</sup> for the 3-OH-C12 HSL and 36.58 nmol·L<sup>-1</sup> for the 3-OH-C14 HSL. Overall, short chain AHLs (with the notable exception of C4-HSL) presented lower LODs compared to long acyl side chain AHLs. All MS/MS fragmentation spectra of the 27 AHL standards can be found in Figure S1 of the Supplementary Material.

### 3.2. Screening of AHL-Producing *Vibrio* Strains

A total of 87 *Vibrio* spp. strains were selected from the BBCC Culture Collection of c.a. 2000 strains. The strains were previously isolated from seawater, crater lake water, sea urchin, green alga, sponge, Rodophyta, macrophytes, Urochordata or jellyfish and from different geographical sites. These strains are closely related to 28 described species, as shown in Table 1. Isolates closely related to the Splendidus clade represented 40% of the strains, the highest number of isolates was related to *V. gigantis* (16 isolates) mostly isolated from benthic and pelagic macro-organisms followed by *V. splendidus* (8 isolates) which were rather isolated from seawater. The 26 remaining species varied between 1 and 7 isolates. A 16S rRNA phylogenetic analysis of the strains and the GenBank accession numbers are provided in the Supplementary Material Figure S2 and Table S1.

In order to detect a broad diversity of AHLs, the isolates were tested for their capability to produce AHLs using three biosensors, *P. putida* F117 (pKR-C12), *E. coli* MT102 (pJBA-132) and *C. violaceum* (CV026; Table 1). The vast majority of the analyzed isolates (91%) did not produced AHLs able to activate any of our three biosensors in the defined experimental conditions. Eight isolates (9%) closely related to the species *V. mytili*, *V. metschnikovii*, *V. scophtalmi*, *V. tasmaniensis* and *V. ordalii* activated at least one of the three bioreporter strains. Strain BBCC 1015 (*V. ordalii*), CIP 107715 (*V. tasmaniensis*, LGP32) and BBCC 2428 (*V. mytili*) activated the biosensor *C. violaceum* CV026. The strain BBCC 1015 (*V. ordalii*), BBCC 1026 and 1055 (*V. metschnikovii*), BBCC 1228, 1237, and 1238 (*V. scophtalmi*), CIP 107715 (*V. tasmaniensis*, LGP32) activated the biosensor *E. coli* MT102. All these strains, except for LGP32, also activated the biosensor *P. putida* F117. In addition to the fact that LGP32 is likely to produce a diverse panel of AHLs, this strain is also a well-known pathogen of marine invertebrates including some with commercial interest [1]. It therefore appeared important to describe the AHLs produced by this strain.

### 3.3. *Vibrio Tasmaniensis* LGP32: AHLs Characterization by UHPLC-HRMS/MS

The culture supernatant was extracted with ethyl acetate and fractionated into 22 fractions, identified as LGP32\_a to LGP32\_v. These fractions were then tested for AHL production using the biosensor strains *E. coli* MT102 and *P. putida* F117. A total of four fractions (LGP32\_1, LGP32\_m, LGP32\_o and LGP32\_p) were positive with at least one of the biosensors. UHPLC-HRMS and UHPLC-HRMS/MS analyses were then performed to identify AHLs. The presence of AHLs in the fractions were revealed by the detection in the fragmentation patterns of at least one of the following characteristic lactone ring fragments (*m/z* 102.055, 84.045, 74.061 and 56.050) [35]. In the condition tested, four different AHLs were detected including unsubstituted, oxo and hydroxy AHLs at the third carbon atom (Table 3). The AHLs detected for this strain were C10-HSL (*N*-decanoyl homoserine lactone), OH-C12-HSL (*N*-3-hydroxy-dodecanoyl homoserine lactone), oxo-C12-HSL

(*N*-3-oxo-dodecanoyl homoserine lactone) and C14:1-HSL (*N*-tetradecenoyl homoserine lactone). These identifications were supported by the analysis of 27 standard AHLs (Table 4), the retention time and the exact mass of the  $[M + H]^+$  pseudo-molecular ion (precision 3 ppm). However, the exact double bond position on the acyl side chain of the C14:1-HSL has not been determined as it is not easily achievable by mass spectroscopy and such determination would have required derivatization methods or high-field NMR.

**Table 3.** UHPLC-HRMS data and AHL identification in *V. tasmaniensis* LGP32. Rt: Retention time. Theoretical mass correspond to the pseudo-molecular ion  $[M + H]^+$ .

Fractions	Rt (min)	Observed Mass	Molecular Formula	Delta ppm	Identification			
					Name	Molecular Formula	Molecular Weight	Theoretical Mass
LGP32_1	10.04	298.2014	C <sub>16</sub> H <sub>28</sub> NO <sub>4</sub>	−0.554	OXO-C12-HSL	C <sub>16</sub> H <sub>27</sub> NO <sub>4</sub>	297.1940	298.2012
LGP32_m	9.82	300.2166	C <sub>16</sub> H <sub>30</sub> NO <sub>4</sub>	2.182	OH-C12-HSL	C <sub>16</sub> H <sub>29</sub> NO <sub>4</sub>	299.2096	300.2169
LGP32_o	9.90	256.1914	C <sub>14</sub> H <sub>26</sub> NO <sub>3</sub>	2.810	C10-HSL	C <sub>14</sub> H <sub>25</sub> NO <sub>3</sub>	255.1834	256.1907
LGP32_p	10.50	310.2383	C <sub>18</sub> H <sub>32</sub> NO <sub>3</sub>	2.158	C14:1-HSL	C <sub>18</sub> H <sub>31</sub> NO <sub>3</sub>	309.2303	310.2376

**Table 4.** UHPLC-HRMS data of AHL Standards. Rt: Retention time. Theoretical mass correspond to the pseudo-molecular ion  $[M + H]^+$ . MS/MS spectra for AHL standards can be found in Supplementary Information (Figure S1).

AHL Standard	Molecular Formula	Theoretical Mass	Observed Mass	Rt (min)
C4-HSL	C <sub>8</sub> H <sub>13</sub> NO <sub>3</sub>	172.0968	172.0968	5.26
C6-HSL	C <sub>10</sub> H <sub>17</sub> NO <sub>3</sub>	200.1281	200.1281	8.43
OXO-C6-HSL	C <sub>10</sub> H <sub>15</sub> NO <sub>3</sub>	214.1074	214.1072	7.56
C7-HSL	C <sub>11</sub> H <sub>19</sub> NO <sub>3</sub>	214.1438	214.1440	8.83
C8-HSL	C <sub>12</sub> H <sub>21</sub> NO <sub>3</sub>	228.1594	228.1594	9.27
OXO-C8-HSL	C <sub>12</sub> H <sub>19</sub> NO <sub>4</sub>	242.1387	242.1381	8.69
OH-C8-HSL	C <sub>12</sub> H <sub>21</sub> NO <sub>4</sub>	244.1543	244.154	8.55
C9-HSL	C <sub>13</sub> H <sub>23</sub> NO <sub>3</sub>	242.1751	242.1748	9.57
C10-HSL	C <sub>14</sub> H <sub>25</sub> NO <sub>3</sub>	256.1907	256.1907	9.90
OXO-C10-HSL	C <sub>14</sub> H <sub>23</sub> NO <sub>4</sub>	270.1700	270.1699	9.43
OH-C10-HSL	C <sub>14</sub> H <sub>25</sub> NO <sub>4</sub>	272.1856	272.1856	9.25
C11-HSL	C <sub>15</sub> H <sub>27</sub> NO <sub>3</sub>	270.2064	270.2063	10.13
C12-HSL	C <sub>16</sub> H <sub>29</sub> NO <sub>3</sub>	284.2220	284.2220	10.46
OXO-C12-HSL	C <sub>16</sub> H <sub>27</sub> NO <sub>4</sub>	298.2013	298.2013	10.04
OH-C12-HSL	C <sub>16</sub> H <sub>29</sub> NO <sub>4</sub>	300.2169	300.2169	9.87
C13-HSL	C <sub>17</sub> H <sub>31</sub> NO <sub>3</sub>	298.2377	298.2377	10.63
C14-HSL	C <sub>18</sub> H <sub>33</sub> NO <sub>3</sub>	312.2533	312.2533	10.93
C14:1-HSL	C <sub>18</sub> H <sub>31</sub> NO <sub>3</sub>	310.2377	310.2370	10.51
OXO-C14:1-HSL	C <sub>18</sub> H <sub>29</sub> NO <sub>4</sub>	324.2169	324.2170	10.23
OXO-C14-HSL	C <sub>18</sub> H <sub>31</sub> NO <sub>4</sub>	326.2326	326.2322	10.56
OH-C14-HSL	C <sub>18</sub> H <sub>33</sub> NO <sub>4</sub>	328.2482	328.2482	10.42
C15-HSL	C <sub>19</sub> H <sub>35</sub> NO <sub>3</sub>	326.2690	326.2689	11.15
C16-HSL	C <sub>20</sub> H <sub>37</sub> NO <sub>3</sub>	340.2846	340.2846	11.34
C16:1-HSL	C <sub>20</sub> H <sub>35</sub> NO <sub>3</sub>	338.2690	338.2704	10.93
OXO-C16:1-HSL	C <sub>20</sub> H <sub>33</sub> NO <sub>4</sub>	352.2482	352.2497	10.61
C18-HSL	C <sub>22</sub> H <sub>42</sub> NO <sub>3</sub>	368.3159	368.3155	11.66
C18:1-HSL	C <sub>22</sub> H <sub>39</sub> NO <sub>3</sub>	366.3003	366.3003	11.40

## 4. Discussion

### 4.1. Diversity of AHL Producing Strains

We investigated AHL production among 87 *Vibrio* spp. strains using three different bioreporter strains. Remarkably, while the production of AHL quorum-sensing signal molecules has been widely reported among *Vibrio*, only a small percentage of our *Vibrio* spp. strains (9%) were shown to produce AHLs.



This result was different from the observations made by Garcia-Aljaro [22] and Purohit et al., [56] who found that the majority (85%) of *Vibrio* spp. strains in their collection were AHL producers. Similarly, Yang et al. focused on 25 strains and found 23 positive for AHL production [31]. By contrast, and in line with our study, Rasmussen et al., found only 32 positive strains (10%) among the 301 tested in their culture collection [24]. Different non-exclusive hypotheses can be made to explain this low percentage of AHL producing *Vibrio*: (1) the growth conditions might not have been optimal for all *Vibrio* spp. strains to reach the necessary density to produce AHLs or the threshold is variable among strains [57]; (2) The concentrations of produced AHLs might be below the limit of detection of our biosensors; (3) The strains produce *novel* or undetected AHLs, that are not activating our biosensors and (4) The strains do not contain the machinery necessary to produce AHLs. Since we followed the culture growth and we did not observe significant differences in OD between positive and negative strains, hypothesis (1) is somewhat less supported by our results than the remainder explanations. The differences between the activation profiles among strains reflect a diversity of produced AHLs and that can be affected by the genetic diversity of AHL synthases but also the presence of one or more synthases in their genome [58], in agreement with previous observations showing high intraspecific genetic diversity in the genus *Vibrio* [59]. However, interactions and ecological processes that drive or are affected by this phenotypic variation are still poorly understood and warrant further studies [60,61].

#### 4.2. AHL Diversity of *Vibrio Tasmaniensis* LGP32

The observation of AHL production in *Vibrio tasmaniensis* LGP32 is notable, as this strain is a well-known pathogen and producer of outer membrane vesicles (OMVs), OmpU, porins and metalloproteases [41,62,63], all involved in the virulence in oyster larvae [44]. We detected four produced AHLs: C10-HSL, 3-OH-C12-HSL, 3-oxo-C12-HSL and C14:1-HSL and this is the first report of C10-HSL, 3-oxo-C12-HSL and C14:1-HSL in *Vibrio* strains belonging to the Splendidus clade. On the other hand 3-OH-C12-HSL have already been reported [24,54]. Among these four AHLs, three have already been identified in the putative pathogens *V. campbellii*, *V. furnissii*, *V. fluvialis* and *V. anguillarum* [32,64–66]. More broadly among Gram-negative *Proteobacteria*, 3-oxo-C12-HSL controls biofilm production in *Pseudomonas aeruginosa* and has a determining immunomodulatory activity of the human host [67], 3-OH-C12-HSL is involved in virulence factor production by *Acinetobacter baumannii* [68], and finally C14:1-HSL participate in the establishment of a necrosis phenotype in *Agrobacterium vitis* [69]. Considering the AHLs regulation of virulence mechanisms in other *Vibrio* species [11,70,71] and other *Proteobacteria*, our results might add to the understanding of the role of AHLs in the physiology and the pathogenicity of these microorganisms.

#### 4.3. Method Performance

In addition to the results described above, our work also provides data on AHL detection limits by AHL bioreporter strains and by UHPLC-HRMS/MS. Such data, and more especially their comparison, is crucial for future studies of AHL production by *Proteobacteria*. Previous reports have already characterized the limits of detection for these biosensors [36,51,53]. However, in this study, we extended our work to a larger panel of AHLs including long acyl side chain compounds (>14 carbons in the acyl chain), that have not been determined before. While numerous studies have demonstrated that the ability to detect OH-HSL is unique to *Agrobacterium tumefaciens* NTL4 (pZLR4) [22,72], we showed that *Pseudomonas putida* F117 is also able to detect that type of AHLs with similar detection limits (between 13.43 and 0.125 nmol·L<sup>-1</sup>).

Our UHPLC-HRMS protocol yielded a good separation and well defined peaks for 26 AHL standards, with a mass accuracy for all standards below 3 ppm and a median LOD of 10.58 nmol·L<sup>-1</sup>. Finally, the combination of two GFP-based biosensors, *E. coli* MT102 and *P. putida* F117, was responsive to over 90% of the AHL standards and exhibited a lower limit of detection than analytical UHPLC-HRMS methods [24,35]. Our protocol included larger culture volumes compared to those used in similar studies (75 µL to 50 mL [24,35,56]), an activity-based screening of HPLC fractions, and

UHPLC-HRMS/MS structural determination that can in theory increase the detection of rarer and/or *novel* AHL structures when compared with these previously published approaches. The simple fact that HPLC fractions are less complex than supernatants or raw extracts might significantly increase the discovery of novel QS-receptor agonists in the future. In the current study, we did not quantify the AHLs production, but this method is fully compatible with quantification.

The non-targeted UHPLC/HRMS/MS method reported here allows, prior to NMR structure determination, the identification of *novel* AHLs without any corresponding standards. The AHL identification is based on the study of MS/MS fragmentation patterns and the search of characteristic fragment ions corresponding to the lactone ring fragmentation [35]. In theory, this method could be more suitable for comparative studies of AHL production. Unfortunately, we did not uncover *novel* AHLs in LGP32 to demonstrate this potential but several ongoing studies with other gram-negative bacterial strains in our group have already yielded *novel* AHL structures using the same approach.

## 5. Conclusions

In this work, we confirmed that AHL are produced by different species of *Vibrio* and that this production varies among different strains of the same species, pointing to the need of further studies to understand the biological origin as well as ecological significance of this intraspecific variation. To our knowledge, this is the first study that demonstrated AHLs production of *Vibrio tasmaniensis* LGP32 a pathogenic bacterium involved in oyster mortality. Four AHLs, namely C10-HSL, OH-C12-HSL, oxo-C12-HSL and C14:1-HSL were detected and identified by our novel approach combining a HPLC fractionation followed by an activity-based AHL identification by UHPLC-HRMS/MS. This study should be useful for the understanding of the virulence and physiology mechanisms of LGP32. In addition, the limit of detection of a large panel of AHL standards was established and a broader response range was highlighted for the biosensors *Escherichia coli* MT102 and *Pseudomonas putida* F117 compared to previous studies.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1424-8220/17/4/906/s1>, Figure S1: Fragmentation MS/MS spectra of AHL standards, Figure S2: Maximum likelihood tree of 16S rDNA gene sequence (559 bp) of the 87 isolates and 35 type strains of *Vibrio* using the Kimura 2 parameter (K2+G+I, Mega), Table S1: List of the 87 tested strains with their origin, their identification by 16S rRNA gene sequence and their GenBank accession numbers.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Austin, B. Vibrios as causal agents of zoonoses. *Vet. Microbiol.* **2010**, *140*, 310–317. [[CrossRef](#)] [[PubMed](#)]
2. Kwok, A.Y.; Wilson, J.T.; Coulthart, M.; Ng, L.-K.; Mutharia, L.; Chow, A.W. Phylogenetic study and identification of human pathogenic *Vibrio* species based on partial hsp 60 gene sequences. *Can. J. Microbiol.* **2002**, *48*, 903–910. [[CrossRef](#)] [[PubMed](#)]
3. Sawabe, T.; Ogura, Y.; Matsumura, Y.; Feng, G.; Amin, A.R.; Mino, S.; Nakagawa, S.; Sawabe, T.; Kumar, R.; Fukui, Y.; et al. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: Proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. *Front. Microbiol.* **2013**, *4*, 414. [[CrossRef](#)] [[PubMed](#)]

4. Chimetto Tonon, L.A.; Silva, B.S.; Moreira, A.P.B.; Valle, C.; Alves, N.; Cavalcanti, G.; Garcia, G.; Lopes, R.M.; Francini-Filho, R.B.; de Moura, R.L.; et al. Diversity and ecological structure of vibrios in benthic and pelagic habitats along a latitudinal gradient in the Southwest Atlantic Ocean. *PeerJ* **2015**, *3*, e741. [[CrossRef](#)] [[PubMed](#)]
5. Vezzulli, L.; Pezzati, E.; Stauder, M.; Stagnaro, L.; Venier, P.; Pruzzo, C. Aquatic ecology of the oyster pathogens *Vibrio splendidus* and *Vibrio aestuarianus*. *Environ. Microbiol.* **2015**, *17*, 1065–1080. [[CrossRef](#)] [[PubMed](#)]
6. Ben-Haim, Y. *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 309–315. [[PubMed](#)]
7. Heidelberg, J.F.; Heidelberg, K.B.; Colwell, R.R. Bacteria of the -subclass *Proteobacteria* associated with zooplankton in Chesapeake bay. *Appl. Environ. Microbiol.* **2002**, *68*, 5498–5507. [[CrossRef](#)] [[PubMed](#)]
8. Soto, W.; Gutierrez, J.; Remmenga, M.D.; Nishiguchi, M.K. Salinity and temperature effects on physiological responses of *Vibrio fischeri* from diverse ecological niches. *Microb. Ecol.* **2009**, *57*, 140–150. [[CrossRef](#)] [[PubMed](#)]
9. Wendling, C.C.; Wegner, K.M. Adaptation to enemy shifts: Rapid resistance evolution to local *Vibrio* spp. in invasive Pacific oysters. *Proc. R. Soc. B Biol. Sci.* **2015**, *282*, 20142244. [[CrossRef](#)] [[PubMed](#)]
10. Hammer, B.K.; Bassler, B.L. Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol. Microbiol.* **2003**, *50*, 101–104. [[CrossRef](#)] [[PubMed](#)]
11. Zhu, J.; Miller, M.B.; Vance, R.E.; Dziejman, M.; Bassler, B.L.; Mekalanos, J.J. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 3129–3134. [[CrossRef](#)] [[PubMed](#)]
12. Eberhard, A.; Burlingame, A.L.; Eberhard, C.; Kenyon, G.L.; Neelson, K.H.; Oppenheimer, N.J. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **1981**, *20*, 2444–2449. [[CrossRef](#)] [[PubMed](#)]
13. Fuqua, W.C.; Winans, S.C.; Greenberg, E.P. Quorum sensing in bacteria: The LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **1994**, *176*, 269–275. [[CrossRef](#)] [[PubMed](#)]
14. Neelson, K.H.; Platt, T.; Hastings, J.W. Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* **1970**, *104*, 313–322. [[PubMed](#)]
15. Bassler, B.L. How bacteria talk to each other: Regulation of gene expression by quorum sensing. *Curr. Opin. Microbiol.* **1999**, *2*, 582–587. [[CrossRef](#)]
16. Henke, J.M.; Bassler, B.L. Bacterial social engagements. *Trends Cell Biol.* **2004**, *14*, 648–656. [[CrossRef](#)] [[PubMed](#)]
17. Miller, M.B.; Bassler, B.L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **2001**, *55*, 165–199. [[CrossRef](#)] [[PubMed](#)]
18. Persat, A.; Nadell, C.D.; Kim, M.K.; Ingremeau, F.; Siryaporn, A.; Drescher, K.; Wingreen, N.S.; Bassler, B.L.; Gitai, Z.; Stone, H.A. The mechanical world of bacteria. *Cell* **2015**, *161*, 988–997. [[CrossRef](#)] [[PubMed](#)]
19. Engebrecht, J.; Silverman, M. Identification of genes and gene products necessary for bacterial bioluminescence. *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 4154–4158. [[CrossRef](#)] [[PubMed](#)]
20. Fuqua, C.; Greenberg, E.P. Self perception in bacteria: Quorum sensing with acylated homoserine lactones. *Curr. Opin. Microbiol.* **1998**, *1*, 183–189. [[CrossRef](#)]
21. Gilson, L.; Kuo, A.; Dunlap, P.V. AinS and a new family of autoinducer synthesis proteins. *J. Bacteriol.* **1995**, *177*, 6946–6951. [[CrossRef](#)] [[PubMed](#)]
22. García-Aljaro, C.; Vargas-Cespedes, G.J.; Blanch, A.R. Detection of acylated homoserine lactones produced by *Vibrio* spp. and related species isolated from water and aquatic organisms. *J. Appl. Microbiol.* **2012**, *112*, 383–389. [[CrossRef](#)] [[PubMed](#)]
23. Meighen, E.A. Bacterial bioluminescence: Organization, regulation, and application of the lux genes. *FASEB J.* **1993**, *7*, 1016–1022. [[PubMed](#)]
24. Rasmussen, B.; Nielsen, K.; Machado, H.; Melchiorson, J.; Gram, L.; Sonnenschein, E. Global and phylogenetic distribution of quorum sensing signals, acyl homoserine lactones, in the family of *Vibrionaceae*. *Mar. Drugs* **2014**, *12*, 5527–5546. [[CrossRef](#)] [[PubMed](#)]
25. Milton, D.L. Quorum sensing in vibrios: Complexity for diversification. *Int. J. Med. Microbiol.* **2006**, *296*, 61–71. [[CrossRef](#)] [[PubMed](#)]
26. Hmelo, L.; van Mooy, B. Kinetic constraints on acylated homoserine lactone-based quorum sensing in marine environments. *Aquat. Microb. Ecol.* **2009**, *54*, 127–133. [[CrossRef](#)]

27. Burton, E.O.; Read, H.W.; Pellitteri, M.C.; Hickey, W.J. Identification of acyl-homoserine lactone signal molecules produced by *Nitrosomonas europaea* strain Schmidt. *Appl. Environ. Microbiol.* **2005**, *71*, 4906–4909. [[CrossRef](#)] [[PubMed](#)]
28. Shaw, P.D.; Ping, G.; Daly, S.L.; Cha, C.; Cronan, J.E.; Rinehart, K.L.; Farrand, S.K. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 6036–6041. [[CrossRef](#)] [[PubMed](#)]
29. Yong, Y.-C.; Zhong, J.-J. A genetically engineered whole-cell pigment-based bacterial biosensing system for quantification of *N*-butyryl homoserine lactone quorum sensing signal. *Biosens. Bioelectron.* **2009**, *25*, 41–47. [[CrossRef](#)] [[PubMed](#)]
30. Steindler, L.; Venturi, V. Detection of quorum-sensing *N*-acyl-homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol. Lett.* **2007**, *266*, 1–9. [[CrossRef](#)] [[PubMed](#)]
31. Yang, Q.; Han, Y.; Zhang, X.-H. Detection of quorum sensing signal molecules in the family *Vibrionaceae*. *J. Appl. Microbiol.* **2011**, *110*, 1438–1448. [[CrossRef](#)] [[PubMed](#)]
32. Buchholtz, C.; Nielsen, K.F.; Milton, D.L.; Larsen, J.L.; Gram, L. Profiling of acylated homoserine lactones of *Vibrio anguillarum* in vitro and in vivo: Influence of growth conditions and serotype. *Syst. Appl. Microbiol.* **2006**, *29*, 433–445. [[CrossRef](#)] [[PubMed](#)]
33. Kalia, V.C. (Ed.) *Quorum Sensing vs. Quorum Quenching: A Battle with No End in Sight*; Springer: New Delhi, India, 2015.
34. Li, X.; Fekete, A.; Englmann, M.; Götz, C.; Rothballer, M.; Frommberger, M.; Buddrus, K.; Fekete, J.; Cai, C.; Schröder, P.; et al. Development and application of a method for the analysis of *N*-acylhomoserine lactones by solid-phase extraction and ultra high pressure liquid chromatography. *J. Chromatogr. A* **2006**, *1134*, 186–193. [[CrossRef](#)] [[PubMed](#)]
35. Patel, N.M.; Moore, J.D.; Blackwell, H.E.; Amador-Noguez, D. Identification of unanticipated and novel *N*-acyl *L*-homoserine lactones (AHLs) using a sensitive non-targeted LC-MS/MS method. *PLoS ONE* **2016**, *11*, e0163469. [[CrossRef](#)] [[PubMed](#)]
36. Wagner-Döbler, I.; Thiel, V.; Eberl, L.; Allgaier, M.; Bodor, A.; Meyer, S.; Ebner, S.; Hennig, A.; Pukall, R.; Schulz, S. Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine *Alphaproteobacteria*. *ChemBioChem* **2005**, *6*, 2195–2206. [[CrossRef](#)] [[PubMed](#)]
37. Cao, J.G.; Meighen, E.A. Purification and structural identification of an autoinducer for the luminescence system of *Vibrio harveyi*. *J. Biol. Chem.* **1989**, *264*, 21670–21676. [[PubMed](#)]
38. Fekete, A.; Frommberger, M.; Rothballer, M.; Li, X.; Englmann, M.; Fekete, J.; Hartmann, A.; Eberl, L.; Schmitt-Kopplin, P. Identification of bacterial *N*-acylhomoserine lactones (AHLs) with a combination of ultra-performance liquid chromatography (UPLC), ultra-high-resolution mass spectrometry, and in-situ biosensors. *Anal. Bioanal. Chem.* **2007**, *387*, 455–467. [[CrossRef](#)] [[PubMed](#)]
39. Wang, J.; Quan, C.; Wang, X.; Zhao, P.; Fan, S. Extraction, purification and identification of bacterial signal molecules based on *N*-acyl homoserine lactones: Extraction, purification and identification of HSLs. *Microb. Biotechnol.* **2011**, *4*, 479–490. [[CrossRef](#)] [[PubMed](#)]
40. Balbi, T.; Fabbri, R.; Cortese, K.; Smerilli, A.; Ciacci, C.; Grande, C.; Vezzulli, L.; Pruzzo, C.; Canesi, L. Interactions between *Mytilus galloprovincialis* hemocytes and the bivalve pathogens *Vibrio aestuarianus* 01/032 and *Vibrio splendidus* LGP32. *Fish Shellfish Immunol.* **2013**, *35*, 1906–1915. [[CrossRef](#)] [[PubMed](#)]
41. Duperthuy, M.; Schmitt, P.; Garzon, E.; Caro, A.; Rosa, R.D.; Le Roux, F.; Lautredou-Audouy, N.; Got, P.; Romestand, B.; de Lorgeril, J.; et al. Use of OmpU porins for attachment and invasion of *Crassostrea gigas* immune cells by the oyster pathogen *Vibrio splendidus*. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 2993–2998. [[CrossRef](#)] [[PubMed](#)]
42. Mateo, D.; Spurmanis, A.; Siah, A.; Araya, M.; Kulka, M.; Berthe, F.; Johnson, G.; Greenwood, S. Changes induced by two strains of *Vibrio splendidus* in haemocyte subpopulations of *Mya arenaria*, detected by flow cytometry with LysoTracker. *Dis. Aquat. Organ.* **2009**, *86*, 253–262. [[CrossRef](#)] [[PubMed](#)]
43. Le Roux, F.; Zouine, M.; Chakroun, N.; Binesse, J.; Saulnier, D.; Bouchier, C.; Zidane, N.; Ma, L.; Rusniok, C.; Lajus, A.; et al. Genome sequence of *Vibrio splendidus*: An abundant planctonic marine species with a large genotypic diversity. *Environ. Microbiol.* **2009**, *11*, 1959–1970. [[CrossRef](#)] [[PubMed](#)]
44. De Decker, S.; Reynaud, Y.; Saulnier, D. First molecular evidence of cross-species induction of metalloprotease gene expression in *Vibrio* strains pathogenic for Pacific oyster *Crassostrea gigas* involving a quorum sensing system. *Aquaculture* **2013**, *392–395*, 1–7. [[CrossRef](#)]



45. MOLA Collection. Available online: <http://collection.obs-banyuls.fr/> (accessed on 20 April 2017).
46. Fagervold, S.K.; Urios, L.; Intertaglia, L.; Batailler, N.; Lebaron, P.; Suzuki, M.T. *Pleionea mediterranea* gen. nov., sp. nov., a gammaproteobacterium isolated from coastal seawater. *Int. J. Syst. Evol. Microbiol.* **2013**, *63*, 2700–2705. [[CrossRef](#)] [[PubMed](#)]
47. Eiler, A.; Bertilsson, S. Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes. *Environ. Microbiol.* **2004**, *6*, 1228–1243. [[CrossRef](#)] [[PubMed](#)]
48. Acinas, S.G.; Sarma-Rupavtarm, R.; Klepac-Ceraj, V.; Polz, M.F. PCR-induced sequence artifacts and bias: Insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl. Environ. Microbiol.* **2005**, *71*, 8966–8969. [[CrossRef](#)] [[PubMed](#)]
49. Tatusova, T.A.; Madden, T.L. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol. Lett.* **1999**, *174*, 247–250. [[CrossRef](#)] [[PubMed](#)]
50. Blast. Available online: [https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\\_TYPE=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) (accessed on 20 April 2017).
51. McClean, K.H.; Winson, M.K.; Fish, L.; Taylor, A.; Chhabra, S.R.; Camara, M.; Daykin, M.; Lamb, J.H.; Swift, S.; Bycroft, B.W.; et al. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **1997**, *143*, 3703–3711. [[CrossRef](#)] [[PubMed](#)]
52. Riedel, K.; Hentzer, M.; Geisenberger, O.; Huber, B.; Steidle, A.; Wu, H.; Høiby, N.; Givskov, M.; Molin, S.; Eberl, L. *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **2001**, *147*, 3249–3262. [[CrossRef](#)] [[PubMed](#)]
53. Winson, M.K.; Swift, S.; Fish, L.; Throup, J.P.; Jørgensen, F.; Chhabra, S.R.; Bycroft, B.W.; Williams, P.; Stewart, G.S. Construction and analysis of luxCDABE-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol. Lett.* **1998**, *163*, 185–192. [[CrossRef](#)] [[PubMed](#)]
54. Andersen, J.B.; Heydorn, A.; Hentzer, M.; Eberl, L.; Geisenberger, O.; Christensen, B.B.; Molin, S.; Givskov, M. gfp-Based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl. Environ. Microbiol.* **2001**, *67*, 575–585. [[CrossRef](#)] [[PubMed](#)]
55. Ravn, L.; Christensen, A.B.; Molin, S.; Givskov, M.; Gram, L. Methods for detecting acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. *J. Microbiol. Methods* **2001**, *44*, 239–251. [[CrossRef](#)]
56. Purohit, A.A.; Johansen, J.A.; Hansen, H.; Leiros, H.-K.S.; Kashulin, A.; Karlsen, C.; Smalås, A.; Haugen, P.; Willassen, N.P. Presence of acyl-homoserine lactones in 57 members of the *Vibrionaceae* family. *J. Appl. Microbiol.* **2013**, *115*, 835–847. [[CrossRef](#)] [[PubMed](#)]
57. Bhedi, C.D.; Prevatte, C.W.; Lookadoo, M.S.; Waikel, P.A.; Gillevet, P.M.; Sikaroodi, M.; Campagna, S.R.; Richardson, L.L. Elevated temperature enhances short to medium chain acyl homoserine lactone production by black band disease associated vibrios. *FEMS Microbiol. Ecol.* **2017**. [[CrossRef](#)] [[PubMed](#)]
58. Tait, K.; Hutchison, Z.; Thompson, F.L.; Munn, C.B. Quorum sensing signal production and inhibition by coral-associated vibrios: Quorum sensing and coral-associated vibrios. *Environ. Microbiol. Rep.* **2010**, *2*, 145–150. [[CrossRef](#)] [[PubMed](#)]
59. Sawabe, T.; Koizumi, S.; Fukui, Y.; Nakagawa, S.; Ivanova, E.P.; Kita-Tsukamoto, K.; Kogure, K.; Thompson, F.L. Mutation is the main driving force in the diversification of the *Vibrio splendidus* clade. *Microbes Environ.* **2009**, *24*, 281–285. [[CrossRef](#)] [[PubMed](#)]
60. Keller, L.; Surette, M.G. Communication in bacteria: An ecological and evolutionary perspective. *Nat. Rev. Microbiol.* **2006**, *4*, 249–258. [[CrossRef](#)] [[PubMed](#)]
61. Platt, T.G.; Fuqua, C. What's in a name? The semantics of quorum sensing. *Trends Microbiol.* **2010**, *18*, 383–387. [[CrossRef](#)] [[PubMed](#)]
62. Binesse, J.; Delsert, C.; Saulnier, D.; Champomier-Verges, M.-C.; Zagorec, M.; Munier-Lehmann, H.; Mazel, D.; Le Roux, F. Metalloprotease vsm is the major determinant of toxicity for extracellular products of *Vibrio splendidus*. *Appl. Environ. Microbiol.* **2008**, *74*, 7108–7117. [[CrossRef](#)] [[PubMed](#)]
63. Vanhove, A.S.; Duperthuy, M.; Charrière, G.M.; Le Roux, F.; Goudenège, D.; Gourbal, B.; Kieffer-Jaquinod, S.; Couté, Y.; Wai, S.N.; Destoumieux-Garzón, D. Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. *Environ. Microbiol.* **2015**, *17*, 1152–1165. [[CrossRef](#)] [[PubMed](#)]



64. Derber, C.; Coudron, P.; Tarr, C.; Gladney, L.; Turnsek, M.; Shankaran, S.; Wong, E. *Vibrio furnissii*: An unusual cause of bacteremia and skin lesions after ingestion of seafood. *J. Clin. Microbiol.* **2011**, *49*, 2348–2349. [[CrossRef](#)] [[PubMed](#)]
65. Haldar, S.; Chatterjee, S.; Sugimoto, N.; Das, S.; Chowdhury, N.; Hinenoya, A.; Asakura, M.; Yamasaki, S. Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an Artemia model. *Microbiology* **2011**, *157*, 179–188. [[CrossRef](#)] [[PubMed](#)]
66. Wang, Y.; Wang, H.; Liang, W.; Hay, A.J.; Zhong, Z.; Kan, B.; Zhu, J. Quorum sensing regulatory cascades control *Vibrio fluvialis* pathogenesis. *J. Bacteriol.* **2013**, *195*, 3583–3589. [[CrossRef](#)] [[PubMed](#)]
67. Williams, P.; Camara, M.; Hardman, A.; Swift, S.; Milton, D.; Hope, V.J.; Winzer, K.; Middleton, B.; Pritchard, D.I.; Bycroft, B.W. Quorum sensing and the population-dependent control of virulence. *Philos. Trans. R. Soc. B Biol. Sci.* **2000**, *355*, 667–680. [[CrossRef](#)] [[PubMed](#)]
68. Bhargava, N.; Singh, S.P.; Sharma, A.; Sharma, P.; Capalash, N. Attenuation of quorum sensing-mediated virulence of *Acinetobacter baumannii* by *Glycyrrhiza glabra* flavonoids. *Future Microbiol.* **2015**, *10*, 1953–1968. [[CrossRef](#)] [[PubMed](#)]
69. Hao, G.; Burr, T.J. Regulation of long-chain *N*-acyl-homoserine lactones in *Agrobacterium vitis*. *J. Bacteriol.* **2006**, *188*, 2173–2183. [[CrossRef](#)] [[PubMed](#)]
70. Frans, I.; Michiels, C.W.; Bossier, P.; Willems, K.A.; Lievens, B.; Rediers, H. *Vibrio anguillarum* as a fish pathogen: Virulence factors, diagnosis and prevention. *J. Fish Dis.* **2011**, *34*, 643–661. [[CrossRef](#)] [[PubMed](#)]
71. Lilley, B.N.; Bassler, B.L. Regulation of quorum sensing in *Vibrio harveyi* by LuxO and Sigma-54. *Mol. Microbiol.* **2000**, *36*, 940–954. [[CrossRef](#)] [[PubMed](#)]
72. Cha, C.; Gao, P.; Chen, Y.-C.; Shaw, P.D.; Farrand, S.K. Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Mol. Plant. Microbe Interact.* **1998**, *11*, 1119–1129. [[CrossRef](#)] [[PubMed](#)]



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