



First evidence of quorum sensing activity in bacteria associated with Antarctic sponges

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Received: 7 February 2017 / Revised: 15 February 2018 / Accepted: 20 February 2018 / Published online: 8 March 2018
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Abstract

Porifera dominate vast areas of the Antarctic shelves and are successfully colonized by bacteria. Quorum sensing (QS) is a cell-to-cell communication system based on bacterial population density that, enabling the coordination of group-based behaviour, plays a critical role in the successful colonization of higher organisms, also driving the formation of biofilm for adhesion to surfaces. In this study, the production of *N*-Acyl homoserine lactones (AHLs), signal molecules involved in the QS mechanism, was examined for 211 Antarctic sponge-associated Gram-negative bacteria. AHL production was screened by using three different AHL biodetection systems, i.e. *Agrobacterium tumefaciens* pZLR4, *Chromobacterium violaceum* CV026 and *Pseudomonas putida* pKR-C12 with optimal sensitivity to moderate-chain (C8–C12), short-chain (C4–C8) and long-chain (\geq C14) AHLs, respectively. 57.8% of tested isolates activated at least one of the monitor systems used and belonged mainly to bacterial genera that are known to be involved in surface colonization by biofilm production. A thin-layer chromatographic assay based on the *A. tumefaciens* reporter system was utilized to determine the AHL profiles of five selected positive isolates. Visible spots on thin-layer chromatography (TLC) plates were produced by *Roseobacter* sp. TB60 and *Psychrobacter* sp. TB67 (both from the sponge, *Anoxycalyx joubini*). The former probably produced *N*-(3-oxohexanoyl)-L-homoserine lactone (similar to the standard 3-oxo-C6-HSL), whereas the isolate TB67 produced molecules that were similar to the standard *N*-butanoyl-homoserine lactone (C4-HSL). The obtained results demonstrated that AHL-based signalling may play a key role in sponge–bacteria interactions also in the Antarctic environment.

Keywords Quorum sensing · *N*-acyl homoserine lactones · Gram-negative bacteria · Sponge-associated bacteria · Antarctica

Introduction

Sponges (phylum Porifera) are one of the most ancient extant multicellular animals and can provide valuable insights into the origin and early evolution of Metazoa (Lavrov and Kosevich 2016). As sessile filter feeders, they are capable of removing bacteria, phytoplankton, algae and other particulate marine matter from the surrounding water by pumping many thousands of litres of water through their aquiferous system (Leys et al. 2011). This system is located in the mesohyl matrix where microorganisms are transferred and can establish as microbial consortia of sponges (Hentschel et al. 2012; Bayer et al. 2014). Bacteria may be symbiotic, specific and permanently associated or merely commensally present. From an ecological perspective, marine sponges provide a protected and nutrient-rich niche where extensive interactions among the diverse microbial populations are fostered and probably inevitable (Mohamed et al. 2008; Mangano

Dedication: To the memory of our beloved Prof. Vivia Bruni.

Michaud L.—Posthumous.

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et al. 2009; Hentschel et al. 2012). Given the dense bacterial communities associated with sponges, cell-to-cell communication systems are involved in regulating the bacterial symbiotic colonization of metazoan organisms (Ruby 1996; Parsek and Greenberg 2000; Fuqua et al. 2001; Taylor et al. 2004; Pérez-Rodríguez et al. 2015). Quorum sensing (QS) is an environmental sensing system adopted by bacteria to regulate population density, social activities and physiological processes, including the formation of biofilm for bacterial adhesion to surfaces (Moghadam et al. 2014). Cell-to-cell communication is achieved by the production of small extracellular signal molecules, such as *N*-acyl homoserine lactones (AHLs). The latter were first discovered during an analysis of the symbiotic association between the marine bacterium *Vibrio fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* (Nealson et al. 1970; Eberhard et al. 1981). To date, relatively little information is available on AHL-based QS systems in marine bacteria associated with higher organisms. For example, Taylor et al. (2004) evidenced AHL production by bacteria associated with marine organisms belonging to different taxa (such as sponges, macroalgae, bryozoans, ascidians and corals). Recently, the draft genome of the sponge-associated *Ruegeria halocynthiae* strain MOLA R1/13b reinforced previous observations on the ability of marine bacteria to communicate using QS in sponge microenvironments, where these cells can be found at high concentrations (Doberva et al. 2014). More recently, Saurav et al. (2016) characterized QS-signalling molecules by a *Paracoccus* isolate from the marine sponge *Sarcotragus* sp. Despite Porifera dominance of vast areas of the Antarctic shelves, the associated prokaryotic communities have only rarely been investigated (Webster et al. 2004; Mangano et al. 2009, 2014; Papaleo et al. 2012). First analyses on the inter-population interactions among Antarctic sponge-associated bacteria (Mangano et al. 2009) revealed that antagonism could act as an effective control of the different bacterial populations inhabiting the sponge tissue, conferring a selective advantage in competition for nutrients and space. Results encouraged us to gain further insight into the communication between bacterial populations (intra-population interactions) associated with Antarctic sponges by examining the production of AHLs by Gram-negative bacteria.

Materials and methods

Sample collection

Specimens (3–5) of the Antarctic sponges *Lissodendoryx (Ectyodoryx) nobilis* (Ridley and Dendy 1886) (LN-AC), *Phorbas glaberrimus* (Topsent 1917) (PG), and *Myxodoryx hanitschi* (Kirkpatrick 1907) (MH) were collected from five sites at Terra Nova Bay (Ross Sea, Antarctica), namely

Adelie Cove (AC; coordinates: 74° 46' 556''S–164° 00' 234''E), Caletta (CAL; coordinates: 74° 45' 113''S–164° 05' 320''E), Gondwana (GW; coordinates: 74° 38' 00.5''S–164° 09' 09.8''E), Road Bay (RB; coordinates: 74° 42.038'S–164° 08.167'E), and Thetys Bay (TB; coordinates: 74° 41.698'S–164° 04' 214''E).

Sponge specimens were treated as previously described by Mangano et al. (2009). Briefly, organisms were immediately washed at least three times with filter-sterilized natural seawater to remove transient and loosely attached bacteria and/or debris. Specimens were then placed into individual sterile plastic bags containing filter-sterilized natural seawater and transported directly to the laboratory at 4 °C for microbiological processing (within 2 h after sampling). A fragment of each specimen was also preserved in 70% ethanol for taxonomic identification.

Bacterial isolation and phylogenetic identification

Bacterial isolation

A central core of the sponge tissue was cut by using an EtOH-sterilized corkborer or a sterile scalpel. The sponge tissue was then aseptically weighed and manually homogenized in 0.22 µm filtered seawater, in a sterile mortar. Tissue extracts were serially diluted (up to 10⁻⁴) by using filter-sterilized seawater (Mangano et al. 2009). Aliquots (100 µL) of each dilution were plated in triplicate on Marine Agar 2216 (MA, Difco). Plates were incubated in the dark at 4 °C for one month. Bacterial colonies grown on MA were isolated at random and streaked at least three times before being considered pure. Bacterial cultures were then maintained in the dark at 4 °C under aerobic conditions.

16S rRNA gene PCR amplification of bacterial isolates

PCR-amplification of the 16S rRNA gene from bacterial isolates was carried out under conditions described earlier (Michaud et al. 2004). Briefly, a single colony of each strain was picked-up with a sterile toothpick from an MA plate, re-suspended in 20 µL of sterile distilled water and lysed by heating at 95 °C for 10 min. Cell lysates were rapidly cooled in ice, briefly centrifuged in a microcentrifuge and used directly for PCR amplification. Amplification of the 16S rRNA gene was performed with an ABI 9600 thermocycler (PE, Applied Biosystems) using the domain Bacteria-specific primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (spanning positions from 8 to 27 in *E. coli* rRNA coordinates) and 1492R (5'-CTACGGCTACCTTGTTACGA-3') (spanning positions from 1492 to 1513 in *E. coli*). The reaction mixtures were assembled at 0 °C and contained 2 µL of DNA (1–10 ng DNA), 2 µL of 10X buffer, 0.6 µL of 50 mM MgCl₂, 0.6 µL of each 10 µM forward and reverse

primer (MWG, Germany), 0.4 μL of 2.5 mM dNTP mix, 0.1 μL of 5U μL^{-1} PolyTaq polymerase (Polymed, Italy) and sterile distilled water to a final volume of 20 μL . Negative controls for DNA extraction and PCR setup (reaction mixture without a DNA template) were also used in every PCR run. The PCR program was as follows: 3 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C and a final extension step of 10 min at 72 °C. The expected size of the PCR product was approximately 1.4 kb.

The results of the amplification reactions were analysed by agarose gel electrophoresis (1%, w/v) in TAE buffer (0.04 M Tris–acetate, 0.02 M acetic acid, 0.001 M EDTA), containing 1 $\mu\text{g mL}^{-1}$ of ethidium bromide.

Sequencing and analysis of 16S rRNA genes

Automated sequencing of the 16S rRNA gene was carried out by cycle sequencing using the dye terminator method. Sequencing was carried out at the Sequencing Service of the MacroGen Laboratory (Korea). The closest relatives of isolates were determined by comparing them to 16S rRNA gene sequences in the NCBI GenBank and the EMBL databases using BLAST, and the “Seqmatch” and “Classifier” programs of the Ribosomal Database Project II (<http://rdp.cme.msu.edu/>). Sequences were further aligned using the program Clustal W (Thompson et al. 1994) to the most similar orthologous sequences retrieved from the database. Each alignment was checked manually and corrected.

Isolates are part of the Italian Collection of Antarctic Bacteria (CIBAN) of the National Antarctic Museum (MNA, www.mna.it) “Felice Ippolito” kept at the University of Messina. They are currently maintained on MA slopes at 4 °C and routinely streaked on agar plates from tubes every six months to control purity and viability. Antarctic strains are also preserved by freezing cell suspensions at – 80 °C in Marine Broth (MB, Difco) to which glycerol (20%, v/v, final concentration) is added.

Nucleotide sequence accession numbers

Nucleotide sequences have been deposited in the GenBank database under the accession nos. KY565535– KY565552.

Screening for acyl-homoserine lactone production

A total of 211 Gram-negative Antarctic isolates were screened for AHL production. Among these, 106 were previously isolated from the Antarctic sponge species *L. nobilis* (31 isolates; from Thetys Bay; LN-TB) (Mangano et al. 2009), *Anoxycalex (Scolymastra) joubini* (Topsent, 1916) (33 isolates; AJ) (Mangano et al. 2009), and *Hemigellius pilosus* (Kirkpatrick, 1907) (42 isolates; HP) (Mangano et al. 2014). Briefly, they were mainly affiliated to the

Gammaproteobacteria (89 isolates), followed by the Alphaproteobacteria (12 isolates) and CF group of Bacteroidetes (5 isolates) (Table 1).

N-acyl-homoserine lactone bioreporter systems

Three bioreporter systems with different sensitivities were used. The *gfp*-based biosensor *P. putida* pKR-C12 (AHL-Pa) is composed of *P. putida* harbouring the *gpf* plasmid pKR-C12. In the presence of exogenous AHLs (best responding to 3-oxo-C12-AHL), green fluorescent cells can be detected under epifluorescence microscope (Riedel et al. 2001). The pigment-based biosensor *C. violaceum* strain CV026 (AHL-Cv) is an AHL-deficient and non-pigmented mutant of *Chromobacterium violaceum*, a Gram-negative bacterium that produces the visible purple pigment violacein (McClellan et al. 1997). In the presence of exogenous AHLs (best responding to C6-AHL), this mutant produces violacein and turns purple. Finally, the β -galactosidase-based biosensor *Agrobacterium tumefaciens* pZLR4 (AHL-At) is composed of *A. tumefaciens* harbouring the pZLR4 plasmid producing a blue colony appearance in the presence of exogenous AHLs (Farrand et al. 2002) (for further details, see Saurav et al. 2017).

The AHL biosensor strains, AHL-Cv and AHL-Pa, were maintained in Luria–Bertani (LB, Sigma) with kanamycin (50 $\mu\text{g mL}^{-1}$) and gentamicin (40 $\mu\text{g mL}^{-1}$), respectively. The biosensor strain AHL-At was grown in Nutrient Agar (NA, Oxoid) with gentamicin (30 $\mu\text{g mL}^{-1}$). All biosensor strains were maintained at 28 °C. All analyses were performed in triplicate.

Well diffusion assay using AHL-At as biosensor

To test the production of *N*-acyl-homoserine lactones, extracts of sterile supernatants from bacterial cultures were screened by the well diffusion assay (Ravn et al. 2001) using the monitor system AHL-At. Isolates were inoculated in MB with pH being adjusted to 6 (due to the instability of AHLs at pH above 8) and incubated at 15 °C (due to the psychrotrophic nature of the isolates) until an OD_{600} of about 0.6–1 was reached. AHLs were extracted from 50 mL of cell-free supernatant which was mixed with an equal volume of ethylacetate containing 0.5% acetic acid. Following thorough mixing, the ethylacetate phase was removed, and the volumes of ethylacetate were evaporated. Finally, extracts were redissolved in 100 μL of ethylacetate.

For the monitor assays, the AHL-At was grown for 24 h at 28 °C in AB medium (Chilton et al. 1974), and 1/10 of the outgrown culture was mixed with 50 mL of melted AB agar containing 20 $\mu\text{g mL}^{-1}$ of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Promega) and poured into Petri dishes. Wells of 6 mm in diameter were punched in the

Table 1 16S rRNA gene sequence affiliation to their closest phylogenetic neighbours of isolates from Antarctic sponges

Next relative by genbank alignment (AN ^a , organism)	RI ^b	AN	Hom ^c (%)	Sponge species ^d					Reference	
				LN-AC	MH	PG	AJ	LN-TB		HP
<i>Alphaproteobacteria</i>										
U14583, <i>Octadecabacter antarcticus</i> 307	TB71	EU237141	98				5			Mangano et al. (2009)
AJ968651, <i>Roseobacter pelophilus</i> SAM4T	TB73	EU237142	98				2			Mangano et al. (2009)
AY167339, <i>Roseobacter</i> sp. ARK9990	TB60	EU237137	96				1			Mangano et al. (2009)
KJ475185, <i>Roseovarius</i> sp. PAMC 27236	TB59	EU237136	100				1			Mangano et al. (2009)
KC987002, <i>Sphingomonas faeni</i> TP-Snow-C72	AC69	KY565535	99	2						This study
DQ781321, <i>Sphingopyxis</i> sp. FR1093	TB79	EU237144	97				2			Mangano et al. (2009)
EU237146, <i>Sphingopyxis</i> sp. TB 82	AC214	KY565536	99			4				This study
AJ534237, <i>Sulfitobacter</i> sp. LM-16	AC234	KY565537	98			1				This study
AJ534237, <i>Sulfitobacter</i> sp. LM-16	TB81	EU237145	99				1			Mangano et al. (2009)
<i>Gammaaproteobacteria</i>										
KP745581, <i>Alivibrio logei</i> strain 27 K.1	TB35	EU237129	99					1		Mangano et al. (2009)
GQ452867, <i>Colwellia</i> sp. BSw21531	GW172	KC709480	99						3	Mangano et al. (2014)
KR012292, <i>Pseudoalteromonas</i> sp. JXH-304 16S	CAL510	KY565539	100	2	10					This study
JF273877, <i>Pseudoalteromonas</i> sp. TB 22	CAL494	KY565544	99		11					This study
HQ534322, <i>Pseudoalteromonas</i> sp. COL-35	AC248	KY565540	98			1				This study
LN881406, <i>Pseudoalteromonas</i> sp. An89	AC167	KY565541	99		12					This study
JX429804, <i>Pseudoalteromonas</i> sp. SS263-28	AC147	KY565542	97	34						This study
JF273874, <i>Pseudoalteromonas</i> sp. TB14	AC62	KY565543	100	10						This study
EF382701, <i>Pseudoalteromonas</i> sp. BSi20430	TB5	EU237121	99					15	5	Mangano et al. (2009)
EF635238, <i>Pseudoalteromonas</i> sp. BSw20679	TB49	EU237134	99				6		8	Mangano et al. (2009)
LN881406, <i>Pseudoalteromonas</i> sp. An89	GW179	KC709471	99						8	Mangano et al. (2014)
AF320989, <i>Pseudomonas tolaasii</i> NCPPB 2193	TB76	EU237143	99				1			Mangano et al. (2009)
KM586864, <i>Psychrobacter</i> sp. BAL309	GW97	KY565545	98			3				This study
KP849536, <i>Psychrobacter</i> sp. A10	CAL495	KY565546	99		1					This study
DQ399762, <i>Psychrobacter</i> sp. B-5161	TB15	EU237123	99					3	6	Mangano et al. (2009)
AJ430827, <i>Psychrobacter fozii</i> LMG 21280	TB55	EU237135	99				10	1	3	Mangano et al. (2009)
AB681355, <i>Psychrobacter</i> sp. NBRC 101054	GW210	KC709475	99						4	Mangano et al. (2014)
JF721975, <i>Psychromonas arctica</i> HQF5	GW94	KY565538	99			3				This study
FJ546071, <i>Shewanella</i> sp. S16-5-4	AC127	KY565547	99	1						This study
KC709473, <i>Shewanella</i> sp. Hp10	AC91	KY565548	99			1				This study
JQ800230, <i>Shewanella</i> sp. KJF13-12	AC34	KY565549	99	2						This study
JF273927, <i>Shewanella</i> sp. AC105	AC74	KY565550	99	2						This study
DQ530472, <i>Shewanella</i> sp. gap-f-53	TB31	EU237128	99					5	2	Mangano et al. (2009)
DQ667116, <i>Shewanella</i> sp. E9	TB36	EU237130	98					6	1	Mangano et al. (2009)

Table 1 (continued)

Next relative by genbank alignment (AN ^a , organism)	RI ^b	AN	Hom ^c (%)	Sponge species ^d					Reference
				LN-AC	MH	PG	AJ	LN-TB	
FJ889553, <i>Shewanella</i> sp. BSs20053	CAL98	KC709476	99					1	Mangano et al. (2014)
KP259764, <i>Vibrio</i> sp. IM4AM	AC289	KY565551	99		1				This study
CFB group of <i>Bacteroidetes</i>									
JQ800200, <i>Bizionia</i> sp. KJF12-3	156	KC709477	99					1	Mangano et al. (2014)
AY771766, <i>Formosa algae</i> clone SE60	TB65	EU237139	100				1		Mangano et al. (2009)
JQ800144, <i>Polaribacter</i> sp. KJF9-6	CAL511	KY565552	98		1				This study
DQ167236, <i>Psychroserpens</i> sp. AKS 190	TB39	EU237131	99				3		Mangano et al. (2009)

MH *Myxodoryx* *M. hantitschi*, HPPG *Phorbas glaberrimus* *H. pilosus*; AJ, *Anoxycalyx joubini*, LN-TB *Lyssodendoryx* *L. nobilis* from Thetys Bay, HP *Hemigellius pilosus*

^aAN, Accession Number

^bRI, isolate representative of each phylotype

^cHom, sequence homology

^dLN-AC *Lyssodendoryx. nobilis* from Adelle Cove

solidified agars, and sample extracts of 50 μ L were pipetted into the wells. Plates with AHL-At were incubated for 16–24 h at 28 $^{\circ}$ C. The development of blue halos, due to AHL-induced β -galactosidase activity around agar wells, was recorded as a positive result. Three distinct “activation” phenotypes were distinguished in marine agar assay: strong activation (++) , moderate activation (+) , weak activation (w) and no activation (–).

Screening for *N*-acyl-homoserine lactones using the AHL-Pa and AHL-Cv monitor systems

AHL-Cv and AHL-Pa were utilized in the T-streak assay to determine the presence of short and long acyl chain AHLs, respectively. Briefly, tester strains were streaked on MA, and plates were incubated at 15 $^{\circ}$ C until satisfactory growth was obtained. The incubation temperature of 15 $^{\circ}$ C was chosen for the screening due to the psychrotrophic nature of tested isolates (Mangano et al. 2009).

The biosensor strain was streaked to form a “T” to the tester, and plates were further incubated at 28 $^{\circ}$ C for 24–48 h. The phenotypic changes associated with the presence of exogenous AHLs were observed at the meeting point of the two strains: the activation of AHL-Cv was revealed by the presence of purple zones due to AHL-induced violacein, while the activation of AHL-Pa was revealed by the expression of the green fluorescent protein (GFP), therefore the presence of fluorescence.

As was described for the use of AHL-At, three distinct “activation” phenotypes were distinguished in marine agar assay: strong activation (++) , moderate activation (+) , weak activation (w) and no activation (–).

Thin-Layer Chromatography profiling of *N*-acyl-homoserine lactones

The biosensor AHL-At was used to visualize the AHLs from selected isolates on thin-layer chromatography (TLC) plates. Briefly, sample extracts of 50 μ L, obtained as described above for the well diffusion assay, were loaded onto C₁₈ TLC plates (TLC silica plates F₂₅₄, 20 \times 20 cm; Merck, Darmstadt, Germany), and standards included on the plates were 3-oxo-AHL, *N*-alkanoyl-AHL and 3-hydroxy-AHL (Sigma Chemicals). Plates were developed in 200 mL of 6:4 (v/v) methanol–distilled water as the mobile phase. For detection of AHLs, the TLC plates were air dried and overlaid with a thin film of 0.7% (w/v) AB medium supplemented with X-gal (65 μ g mL⁻¹) and 1/10 of an exponential suspension of the AHL-At reporter. TLC plate overlays were placed in a sealed container and incubated at 28 $^{\circ}$ C for 24 h.

Each AHL migrates with a characteristic mobility, and the position and shape of the sample AHL spots can be

compared with the spots of different standards in order to identify them.

Results

Bacterial strains

Overall, 105 strains were freshly isolated: 53 from *L. nobilis*, 35 from *M. hanitschi*, and 17 from *P. glaberrimus*. All sequences with similarity $\geq 97\%$ were considered to represent one phylotype. A total of 18 phylotypes were obtained, with 6 of them represented by a single isolate (i.e. isolates AC91, AC234, AC248, AC289, CAL495, and CAL511) (as reported in Table 1). The majority of phylotypes were retrieved from single samples, whereas only two phylotypes were shared between different sponge species.

Bacterial isolates were predominantly affiliated to the Gammaproteobacteria (97 isolates), in addition to few members of the Alphaproteobacteria (7 isolates) and CF group of Bacteroidetes (1 isolate). In particular, the Gammaproteobacteria contained 26 phylotypes distributed among 8 genera of the phylum, as follows: *Pseudoalteromonas* (80 isolates in 6 phylotypes), *Shewanella* (9 isolates in 4 phylotypes), *Psychrobacter* (4 isolates in 2 phylotypes), *Psychromonas* (3 isolates in a single phylotype) and *Vibrio* (1 isolate). The three phylotypes within the Alphaproteobacteria were affiliated to the genera *Sphingomonas* (2 isolates), *Sphingopyxis* (4 isolates) and *Sulfotobacter* (1 isolate), respectively. The unique sequence affiliated to the CFB group of Bacteroidetes fell into the genus *Polaribacter*.

Bacterial strains previously isolated were within the Gammaproteobacteria (110 strains), Alphaproteobacteria (9 strains) and CFB group of Bacteroidetes (3 strains) (Mangano et al. 2009, 2014).

Screening for *N*-acyl-homoserine lactone production

A total of 211 Antarctic bacterial isolates (Table 1) from five different sponge species were screened for AHL production. Among them, 122 isolates were able to activate at least one of the monitor systems used for the screening, with only two of them activating all the reporters (Table 2). The AHL-At reporter, which is the most sensitive among those tested, was activated by 116 bacterial strains (out of 211 tested isolates; 54.9%) with five of them that gave a better response: *Roseobacter* sp. TB60 and *Psychrobacter* sp. TB67 from *A. joubini*, *Aliivibrio* sp. TB35 and *Pseudoalteromonas* TB5 from *L. nobilis*, and *Psychrobacter* sp. GW210 from *H. pilosus*.

Due to weak bacterial growth during the experiment, AHL-Pa and AHL-Cv were tested for a selection of isolates

(151 and 64 isolates, respectively). Among them, 17 isolates (11.2% of tested isolates) activated AHL-Pa for short chain AHLs, with *Roseobacter* sp. TB60 from *A. joubini*, *Aliivibrio* sp. TB35 from *L. nobilis*, *Shewanella* spp. CAL98 and CAL102 from *H. pilosus* that were particularly active. Finally, only five strains (7.8% of tested isolates) produced short-chain AHLs, as was demonstrated by the activation of the AHL-Cv reporter, as follows: *Roseobacter* sp. TB 60 and *Octadecabacter* sp. TB71 from *A. joubini*, *Aliivibrio* sp. TB35, *Shewanella* sp. TB36 and *Pseudoalteromonas* sp. TB34 from *L. nobilis*.

Profiling of *N*-acyl-homoserine lactone production by thin-layer chromatography

Isolates that strongly activated the AHL-At biomonitor system were selected for further analyses by TLC: *Aliivibrio* sp. TB35 and *Pseudoalteromonas* sp. TB5 from *L. nobilis*, *Psychrobacter* sp. GW210 from *H. pilosus*, *Roseobacter* sp. TB60 and *Psychrobacter* sp. TB67 from *A. joubini*. Visible spots on TLC plates were produced only by these two latter isolates. In particular, *Roseobacter* sp. TB60 probably produced *N*-(3-oxohexanoyl)-L-homoserine lactone (similar to the standard 3-oxo-C6-HSL), whereas *Psychrobacter* sp. TB67 produced molecules that were similar to the standard *N*-butanoyl-homoserine lactone (C4-HSL) (data not shown).

Discussion

Sponge–microbe associations have been described in several tropical and temperate regions, whereas knowledge about sponge-associated microbial communities in Antarctica remains quite scarce and fragmentary, even though sponges dominate vast areas of the Antarctic shelves. A unique work by Webster et al. (2004) analysed, by culture-independent methods, the entire prokaryotic community associated with five Antarctic sponge species, different from those considered in the present study. Additionally, the cultivable fraction of the associated bacterial community was seldom assayed for heavy metal and antibiotic tolerance (Mangano et al. 2014), antibiotic production for future biotechnological applications (Lo Giudice et al. 2007; Papaleo et al. 2012, 2013; Maida et al. 2015) and inhibitory inter-population interactions (Mangano et al. 2009). As a result of the paucity of knowledge on sponge associated microbial communities in Antarctica, this study was aimed at surveying, for the first time, the production of *N*-acyl-homoserine lactones by Gram-negative bacteria from five Antarctic sponge species. Marine sponges have developed highly specific relationships with numerous associated microorganisms that can amount to 40% of the biomass of the animal (Vacelet 1975; Taylor et al. 2004). Bacteria growing in enclosed niches may use

Table 2 16S rRNA gene sequence affiliation to their closest phylogenetic neighbours of Antarctic isolates that were able to activate at least one of the monitor systems used in this study

Phylum or class ^a	Sponge ^b	Isolate(s)	AHL monitor system ^{b, c}		
			AHL-At	AHL-Pa	AHL-Cv
ALF					
	A. joubiniAJ	<i>Octadecabacter</i> sp. TB72	+	–	–
	AJA. joubini	<i>Octadecabacter</i> sp. TB80	(+)	–	–
	AJA. joubini	<i>Octadecabacter</i> sp. TB71	–	(+)	(+)
	AJA. joubini	<i>Roseobacter</i> sp. TB44	+	–	–
	AJA. joubini	<i>Roseobacter</i> sp. TB73	(+)	–	–
	AJA. joubini	<i>Roseobacter</i> sp. TB60	++	++	++
	AJA. joubini	<i>Roseovarius</i> sp. TB59	+	–	–
	AJA. joubini	<i>Sphingopyxis</i> sp. TB79	(+)	–	–
	P. glaberrimusPG	<i>Sulfitobacter</i> sp. AC234	(+)	–	nd
GAM					
	L. nobilisLN	<i>Aliivibrio</i> sp. TB35	++	++	+
	H. pilosusHP	<i>Colwellia</i> sp. GW172	–	+	nd
	HPH. pilosus	<i>Colwellia</i> sp. GW163	(+)	+	nd
	HPH. pilosus	<i>Colwellia</i> sp. GW185	+	–	nd
	LNL. nobilis	<i>Marinobacter</i> sp. TB3	(+)	–	nd
	LNL. nobilis	<i>Pseudoalteromonas</i> sp. AC119	(+)	–	nd
	M. hanitschiMH	<i>Pseudoalteromonas</i> sp. RB2	(+)	–	nd
	HPH. pilosus	<i>Pseudoalteromonas</i> sp. GW179	(+)	–	nd
	M. hanitschiMH	<i>Pseudoalteromonas</i> spp. CAL494, CAL496	+	–	nd
	HPH. pilosus	<i>Pseudoalteromonas</i> spp. 135, 143, 158, 160, 168, CAL107, GW168, GW170, GW174, GW184, GW186, GW188, GW189, GW191, GW193	+	–	nd
	PGP. glaberrimus	<i>Pseudoalteromonas</i> sp. AC248	+	–	nd
	M. hanitschiMH	<i>Pseudoalteromonas</i> sp. RB1	+	–	nd
	LNL. nobilis	<i>Pseudoalteromonas</i> sp. TB5	++	–	–
	A. joubiniAJ	<i>Pseudoalteromonas</i> spp. TB42, TB43, TB51, TB64	+	–	–
	LNL. nobilis	<i>Pseudoalteromonas</i> spp. TB9, TB10, TB12, TB14, TB17, TB19, TB22, TB24, TB25, TB27, TB30, TB32	+	–	–
	LNL. nobilis	<i>Pseudoalteromonas</i> sp. TB34	+	–	+
	LNL. nobilis	<i>Pseudoalteromonas</i> sp. TB6	(+)	–	–
	AJA. joubini	<i>Pseudoalteromonas</i> sp. TB49	(+)	–	–
	HPH. pilosus	<i>Pseudoalteromonas</i> sp. GW196	+	(+)	nd
	HPH. pilosus	<i>Pseudoalteromonas</i> spp. 148, GW201	–	(+)	nd
	LNL. nobilis	<i>Pseudoalteromonas</i> spp. AC29, AC141, AC147, AC148, AC258	(+)	nd	nd
	LNL. nobilis	<i>Pseudoalteromonas</i> spp. AC133, AC134, AC135, AC137, AC138, AC139, AC140, AC142, AC143, AC144	+	nd	nd
	AJA. joubini	<i>Pseudomonas</i> sp. TB76	+	–	–
	AJA. joubini	<i>Psychrobacter</i> sp. TB67	++	–	–
	LNL. nobilis	<i>Psychrobacter</i> spp. TB15, TB20, TB33	+	–	–
	AJA. joubini	<i>Psychrobacter</i> spp. TB40, TB47, TB56, TB57, TB58, TB61, TB66	+	–	–
	LNL. nobilis	<i>Psychrobacter</i> sp. TB2	(+)	–	–
	A. joubiniAJ	<i>Psychrobacter</i> sp. TB55	(+)	–	–
	HPH. pilosus	<i>Psychrobacter</i> spp. 146, 149, GW166, GW167, GW203, GW205	+	+	nd
	M. hanitschiMH	<i>Psychrobacter</i> sp. CAL495	+	–	nd
	HPH. pilosus	<i>Psychrobacter</i> spp. 154, 169, CAL100, GW202, GW208, GW209	+	–	nd
	HPH. pilosus	<i>Psychrobacter</i> sp. GW210	++	–	nd
	PGP. glaberrimus	<i>Shewanella</i> sp. AC91	–	+	nd
	LNL. nobilis	<i>Shewanella</i> spp. TB7, TB8, TB11, TB29, TB31	(+)	–	–

Table 2 (continued)

Phylum or class ^a	Sponge ^b	Isolate(s)	AHL monitor system ^{b, c}		
			AHL-At	AHL-Pa	AHL-Cv
	LNL. nobilis	<i>Shewanella</i> spp. TB4, TB21, TB28, TB37	+	–	–
	LNL. nobilis	<i>Shewanella</i> sp. TB36	+	–	+
	HPH. pilosus	<i>Shewanella</i> sp. CAL102	(+)	++	nd
	HPH. pilosus	<i>Shewanella</i> sp. CAL98	–	++	nd
	LNL. nobilis	<i>Shewanella</i> sp. AC263	(+)	nd	nd
	LNL. nobilis	<i>Shewanella</i> sp. TB1	(+)	–	–
	HPH. pilosus	<i>Shewanella</i> sp. CAL109	+	–	nd
	PGP. glaberrimus	<i>Vibrio</i> sp. AC289	+	–	nd
BAC	AJA. joubini	<i>Psychroserpens</i> spp. TB45, TB50	+	–	–
	A. joubiniAJ	<i>Formosa</i> sp. TB65	+	–	–

^aALF Alphaproteobacteria, GAM Gammaproteobacteria, BAC CF group of Bacteroidetes

^bAJ *Anoxycalyx joubini*, PG *Phorbis glaberrimus*, LN *Lyssodendoryx nobilis*, HP *Hemigellius pilosus*, MH *Myxodoryx hanitschi*

^{b, c}Activation of the monitor systems: (+) weak activation, + moderate activation, ++ strong activation, – no activation, nd not determined. Monitor systems: AHL-At *Agrobacterium tumefaciens*, AHL-Pa *Pseudomonas putida* pKR-C12, AHL-Cv *Chromobacterium violaceum* CV026

AHL-QS systems. Signal molecules do not diffuse away and concentrate up to threshold levels. Furthermore, inside a host, lactones are less subjected to pH changes, which could cause hydrolysis. Thus, the sponge mesohyl represents an ideal environment for the development of cell-to-cell communication systems. Moreover, the cell–cell communication based on the production of AHLs is often involved in specific aspects of bacterial colonization of metazoan organisms, including biofilm formation, and symbiotic interactions between bacteria and their hosts might be modulated by AHL quorum sensing (Fuqua et al. 2001; Mohamed et al. 2008; Hughes and Sperandio 2008).

A large number of AHL QS systems can be identified by methods based on the use of bacterial biosensor systems. These biosensors do not produce AHLs and contain a functional response–regulator protein cloned together with a cognate target promoter, which positively regulates the transcription of a reporter gene (e.g. bioluminescence, β -galactosidase, green-fluorescent protein and violacein pigment production; Steindler and Venturi 2007; Saurav et al. 2017). The utilization of three biomonitor systems, with a different sensitivity, for the screening of sponge-associated Antarctic isolates led us to identify a high percentage of AHL producers (57.8% of screened isolates), mainly isolated from *L. nobilis*, *H. pilosus* and *A. joubini*. This observation is not to be considered as a definitive trend due to the non-homogeneous number of isolates obtained *per* sponge species. However, AHL production appears to be a common feature in Antarctic sponge-associated bacteria and could be related to the probable associated lifestyle of tested bacterial isolates. The overall incidence of AHL producers within the bacterial community associated with Antarctic sponges

could be higher than here reported, as the culture conditions and sensor strains utilized could influence obtained results. In laboratory-scale experiments, it is quite difficult to reproduce all the biotic and abiotic features which characterize an environment, due to the well-known biases deriving from isolation and cultivation procedures. In fact, it should be pointed out that various environmental factors and specific sponge biology properties (e.g., difference in morphology, structure, host-released cues and nutritional status), as well as the occurrence of other kinds of microbial interactions (e.g. commensalisms and symbiosis, in which also uncultivable bacteria might be involved) affect actual bacterial interactions and certainly influence AHL synthesis in nature (Mohamed et al. 2008). Nevertheless, results obtained in artificial systems could give some precious preliminary indications on bacterial behaviour in a natural environment.

AHL-based QS is very common in Proteobacteria. Interestingly, in this study, most AHL producers belonged to genera (e.g. *Pseudoalteromonas*, *Shewanella* and *Roseobacter*) that have frequently been reported in association with surfaces, and are able to produce biofilm (Brian-Jaisson et al. 2014; Martín-Rodríguez et al. 2014; Zeng et al. 2017). With regard to sponge-associated bacteria, Mohamed et al. (2008) reported that sponge-associated AHL producers were mainly members of the Alphaproteobacteria. Krick et al. (2007) demonstrated the production of several AHLs by a *Mesorhizobium* sp. associated with the sponge *Phakellia ventilabrum* from the Korsfjord (Norway). Mohamed et al. (2008) reported on the production of AHLs by strains isolated from the marine sponges *Mycale laxissima* and *Ircinia strobilina* (Conch Reef, Florida) and related to the *Silicibacter-Ruegeria* subgroup of the

Roseobacter clade. The predominance of members of the *Roseobacter* clade among alphaproteobacterial AHL producers was not surprising (Martens et al. 2007; Cude and Buchan 2013). These bacteria are frequently associated with surfaces and are able to exploit cell–cell communication and interaction, including the production of AHLs, to enhance collective behaviours within the colonizing community (Cude and Buchan 2013). Thus, they may play a key role in shaping the biofilm community structure (Dang et al. 2008). Furthermore, studies on bacterial symbionts of marine sponges suggest that roseobacters are the primary producers of AHLs in these systems (Taylor et al. 2004; Zan et al. 2011). Gammaproteobacterial isolates that activated monitor systems were also identified among genera (e.g. *Vibrio*, *Pseudomonas*, *Pseudoalteromonas*) that have been previously reported in relation to QS ability and, in some cases, biofilm formation and bioluminescence production. With regard to this, *Pseudoalteromonas* spp. were abundant within the cultivable bacterial communities associated with Antarctic sponges. Such a result may depend on the metabolic versatility of Gammaproteobacteria, as well as the competitive advantage probably gained by *Pseudoalteromonas* members thanks to both QS ability and well-known inhibitory activity. This latter was previously observed also for Antarctic isolates (Lo Giudice et al. 2007; Mangano et al. 2009; Papaleo et al. 2012, 2013; Maida et al. 2015). AHL-like activity was also found in Bacteroidetes isolates, confirming previous observations on the production of the same signal molecules by bacteria beyond the Proteobacteria, and reinforcing the ecological significance of AHL-mediated QS processes (Wagner-Dobler et al. 2005). As suggested by several authors, horizontal transfer may have played an important role in the distribution of QS genes (Lerat and Moran 2004; Romero et al. 2010).

The utilization of the most sensitive and least specific AHL biodetection system available (AHL-At; Mohamed et al. 2008) led us to identify most AHL producers among sponge-associated bacteria. However, further analyses were carried out on isolates that were also positive for long-chain and/or short-chain AHL syntheses in the AHL-Pa and AHL-Cv assays, respectively (Mohamed et al. 2008). TLC analysis was performed for five isolates that strongly activated the AHL-At biomonitor system. The TLC profiles were similar to each other, with AHLs that showed three species which separated on the TLC plate: C4- e, C6-AHLs and 3OC6-HSL. The occurrence of these medium-chain-length AHLs is a common phenomenon in the marine environment due to their sensitivity to pH changes. Being highly labile at basic pHs, such as that of seawater, these signal molecules (particularly those with 3-oxo groups or very short acyl chains) may be involved in communication among bacteria enclosed in a niche.

Conclusions

Extreme cold environments, like Antarctica, are exposed to unique environmental characteristics resulting from biotic interactions, such as predation and competition, and abiotic factors, such as seasonality and ice-scouring. Thus, it is possible to suppose that ecological factors may drive chemical mechanisms, like the QS system, in marine microorganisms as a survival response. Porifera provide an important habitat for aquatic microbes that colonize outer surfaces and interstices of ostia and oscula. Symbiotic microorganisms can adhere to the sponge surface by forming biofilm with complex 3D-structures composed of consortia of microbial species encased in extracellular polymeric substances. Thus, it is plausible to assume that bacterial cell-to-cell communication occurs, probably by the production of AHL-type QS signals by a number of bacterial species that are known to be involved in surface colonization by biofilm production (Brian-Jaisson et al. 2014; Martín-Rodríguez et al. 2014; Zeng et al. 2017). Obtained results demonstrated that AHL-based signalling may play a key role in sponge–bacteria interaction and in profiling the associated bacterial community also in the Antarctic environment. Future investigation will be needed to elucidate not only the chemical nature of signal molecules, but also to better understand the AHL-regulation mechanisms involved in symbiotic interactions between sponges and their bacterial communities.

Acknowledgments The authors wish to thank Vittorio Venturi and Laura Steindler (International Centre for Genetic Engineering, Trieste (Italy) for kindly providing the biomonitor systems used in this study, and for their professional support to S. Mangano during her stay in Trieste. We are very grateful also to three anonymous reviewers for their valuable comments and suggestions to improve the manuscript. The authors also thank Mrs Trays Ricciardi who edited the manuscript for improving English language. This work was supported by grants from the PNRA (Programma Nazionale di Ricerche in Antartide), the Italian Ministry of Education and Research (PEA 2004, Research Projects PNRA 2004/1.6 and PNRA16_00020) and the MNA (Museo Nazionale dell'Antartide).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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