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Cyclic Dipeptides Produced by Marine Sponge-Associated Bacteria as Quorum Sensing Signals

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Four bacterial strains belonging to the genera *Vibrio*, *Pseudoalteromonas* and *Photobacterium* were isolated from the marine sponges *Dysidea avara* and *Geodia cynodium*. A *Bacillus* strain was isolated from *Ircinia variabilis*. A screening of molecules involved in quorum sensing (QS) was carried out by TLC-overlay and a new "plate T-streak" test. To analyze quorum quenching (QQ), a plate T-streak was performed with *Chromobacterium violaceum*. Strains of *Vibrio* isolated from both marine sponges and a strain of *Photobacterium* isolated from *G. cynodium*, activated QS bioreporters. A strain of *Pseudoalteromonas* isolated from *D. avara* showed QQ activity. Finally, it is reported that cyclic dipeptides isolated from strains of *Vibrio* sp. and *Bacillus* sp. (isolated from *D. avara* and *I. variabilis*, respectively) were involved in the QS mechanism. The simultaneous presence of bacteria that showed contrasting responses in bioassays for QS signal molecule synthesis in marine sponges could add an interesting dimension to the signalling interactions which may be happening in sponges.

Keywords: Quorum sensing, Diketopiperazines, Plate "T" streak bioassay, Inter-kingdom cross talking.

Marine microorganisms are of considerable current interest as a new and promising source of biologically active compounds [1]. Despite this observation, relatively little attention has been directed toward the study of natural products from marine microorganisms, which are difficult to isolate and cultivate, because only a small percentage of bacterial cells in marine samples ultimately grow under standard culture conditions [2].

The surfaces and internal spaces of marine sponges are a unique microhabitat in which micro-organisms are regularly observed. These environments are more nutrient rich than seawater and most sediments, thus they are a unique niche for the isolation of diverse bacteria and fungi. In fact, high microbial diversity occurs within marine sponges, and interesting "host-symbiont" distributions of these bacterial populations have been discovered [3].

It appears that a given species of sponge contains a mixture of generalist and specialist microorganisms and that the associated microbial communities are fairly stable in both space and time [4]. To date, the roles bacteria play in sponge biology and ecology have not been elucidated, and few reports describe the contribution of symbiotic microbes to sponge well-being or survival [5]. Generally, little is known about the mechanisms that regulate the association and communication between sponges and associated microorganisms. Recently, it has been demonstrated that multispecies bacterial communities can communicate with each other via small secreted molecules, such as *N*-acyl homoserine lactones (AHLs) and diketopiperazines (DKPs), in a process called quorum sensing (QS) [6,7].

The production of AHLs by marine sponge-associated bacteria has been reported [8]. Several DKPs were isolated from an α-*Proteobacterium* of the genus *Ruegeria* associated with the marine sponge *Suberites domuncula* and from strains of the genera *Staphylococcus* and *Bacillus* associated with *Ircinia variabilis*, and it was proposed that these cyclic dipeptides might play a role in QS mechanisms and could regulate bacterial-sponge interactions [9,10]. Due to their rigid structure, chiral nature, and varied side chains, DKPs have been of research interest for their diverse bioactivities; in particular, DKPs isolated from the sponge *Callyspongia* sp. showed inflammatory activity as macrophage cytokine stimulators [11].

In this paper we report the isolation of four bacterial strains associated with marine sponges and the detection of QS molecules that could regulate the interaction between the microbial community and its host. Furthermore it is reported that the cyclic dipeptides, cyclo-(L-prolyl-L-leucine) and cyclo-(L-prolyl-L-phenylalanine), isolated from the marine bacteria *Vibrio* sp. associated with the marine sponge *Dysidea avara*, and cyclo-(*cis*-4-hydroxy-D-prolyl-L-leucine) isolated from *Bacillus* sp. associated with the marine sponge *I. variabilis*, could be involved in QS mechanism.

Specimens of *D. avara* and *Geodia cynodium* sponges, collected in the gulf of Naples, were used for the isolation of bacteria. Among all isolated strains, just *Pseudoalteromonas* sp. associated with *D. avara* was isolated from sponges previously treated with Bactomarine supplemented with a pool of antibiotics.

Strains were identified by PCR analysis as belonging to the genera *Vibrio* and *Pseudoalteromonas* for those associated with *D. avara* and *Vibrio* and *Photobacterium* for those associated with *G. cynodium*. The *Bacillus* sp. strain associated with the marine sponge *I. variabilis*, has been previously isolated [10].

All isolated strains were grown in Bactomarine medium at 30°C with shaking. The growth was monitored by O.D. measurement at 540 nm. After 24 h, all bacteria reached stationary phase. Even though *Pseudoalteromonas* sp. was isolated by using Bactomarine supplemented with a pool of antibiotics (details in *experimental section*), it was susceptible to all antibiotics used in the isolation step (Table 1). This could be evidence that these bacteria have an intracellular localization in sponges which limits exposure to the pool of antibiotics used in the isolation step.

Table 1: Growth assessment of *Pseudoalteromonas* sp. both in Bactomarine (MB) and MB supplemented with antibiotics.

	Pseudoalteromonas sp. in Bactomarine	*Pseudoalteromonas* sp. in Bactomarine supplemented with antibiotics
	O.D. (540 nm)	O.D. (540 nm)
T_0	0,108±0,011*	0,106±0,002*
T_{12h}	0,780±0,050*	0,202±0,003*
T_{24h}	1,318±0,023*	0,248±0,006*
T_{36h}	1,416±0,016*	0,198±0,002*
T_{48h}	1,419±0,002*	0,108±0,001*

* data are reported ± ST.DEV value calculated on three measurements.

All isolated strains were tested for AHL production by means of a TLC-overlay assay with *Agrobacterium tumefaciens* NTL4 and a Lux screen assay. The Dichloromethane extract of cell-free medium of *Vibrio* sp. isolated from *D. avara* showed activation of QS both in a TLC overlay test (Figure 1), and Lux screen assay (Table 2).

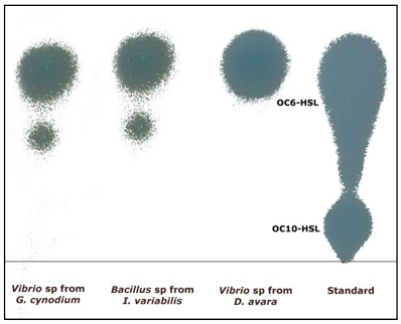

Figure 1: TLC overlay test on cell-free medium dichloromethane extracts of *Vibrio* sp. isolated from *G. cynodium* and *D. avara* and *Bacillus* sp. isolated from *I. variabilis*. Standards: 2 μL of OC_6-HSL (10 μM) and OC_{10}-HSL (100 μM).

Table 2: Lux - screen assay for AHL production.

	Bioreporters		
	pSB1075	pSB401	pSB536
AHL standard[a]	++	++	++
DCM Ex. blank medium[b]	-	-	-
DCM Ex. *Vibrio* sp. from *D. avara*[c]	-	+	-
DCM Ex. *Pseudoalteromonas* sp. from *D. avara*[c]	-	-	-
DCM Ex. *Vibrio* sp. from *G. cynodium*[c]	-	+	-
DCM Ex. *Photobacterium* sp. from *G. cynodium*[c]	-	+	-
DCM Ex. *Bacillus* sp. from *I. variabilis*[c]	-	+	-

[a] AHL standard (C4-HSL for pSB536; C6-HSL for pSB401; C10-HSL for pSB1075);
[b] dichloromethane extracts (DCM Ex.) of blank medium (without inoculum);
[c] dichloromethane extracts of cultural media of marine strains.

Pseudoalteromonas sp. isolated from *D. avara* showed inhibition of QS in a "T" streak assay with *Chromobacterium violaceum*: a negative gradient of violacein production that is under the control of AHL molecules was observed (Figure 2). This means either inhibition or degradation of AHL by *Pseudolateromonas* sp.

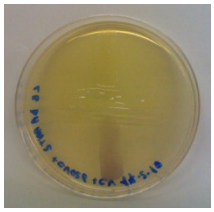

Figure 2: Plate T-streak with *C. violaceum*. quorum quenching activity by *Pseudoalteromonas* sp. isolated from *D. avara*.

The dichloromethane extract of the culture of *Bacillus* sp. showed activation of QS both in TLC overlay and Lux screen assays. Following the positive response in QS bioassays, we then performed a purification of that extract with the aim of isolating compounds responsible for QS induction. The isolated cyclic dipeptide, cyclo-(cis-4-hydroxy-D-prolyl-L-leucine), activated the QS mechanism in *A. tumefaciens* NTL4 biosensor starting at an amount of 0.5 mg (data not shown).

The *Vibrio* strains showed positive responses in the TLC overlay test (Figure 1) and Lux screen assay (Table 2). The positive response was also evident in a new "T" streak assay using double medium. In the standard T-streak assay the tester strain and the biosensor are streaked and grown on the same solid medium to form a "T". Because of the specificity of growth conditions of marine bacteria, we developed a new method in which we prepared double media plates (Bactomarine + ATGN), thus the tester strains and the biosensor (*A. tumefaciens* NTL4) could be streaked on the appropriate medium (details in *experimental section*). We observed the appearance of a blue strip at the meeting point of the two strains/media (Figure 3).

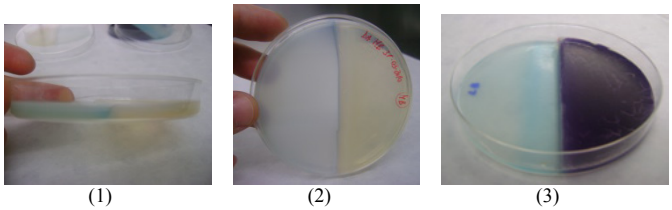

Figure 3: New "T" streak assay (double medium). A blue strip is visible at the interface between the two strains/media: the tester strain (right side) and the bioreporter (*A. tumefaciens* NTL4; left side). (1) *Vibrio* sp. from *G. cynodium* ; (2) *Vibrio* sp. from *D. avara* ; (3) CV-NTL4 (control).

To identify the AHL bioreporter activating signal, the dichloromethane extract (635 mg) of cell-free medium of *Vibrio* sp. (from *D. avara*) was chromatographed on a Lobar RP-18 column eluted with a gradient of H_2O/CH_3OH. Fraction DAMBF5 (260 mg, eluted with H_2O/MeOH 6:4) showed activity in the TLC-overlay bioassay, and was further chromatographed on a Lobar RP-18 column. Four compounds were isolated and chemically characterized by NMR analysis (^1H, ^{13}C).

The isolated compounds showed typical spectra of cyclic dipeptides and were identified as cyclo-(L-prolyl-L-phenylalanine) (**1**), cyclo-(*cis*-4-hydroxy-D-prolyl-L-leucine) (**2**), cyclo-(*trans*-4-hydroxy-L-prolyl-L-phenylalanine) (**3**), cyclo-(L-prolyl-L-leucine) (**4**). The spectral data were in accordance with those reported in the literature [10].

Of the purified cyclic dipeptides, compounds **4** and **1** activated the CV026 bioreporter, starting at an amount of 0.5 mg (Figure 4).

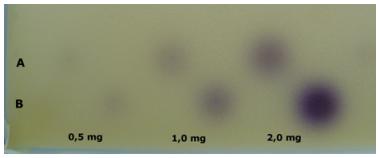

Figure 4: TLC-overlay performed as spot test with biosensor CV026 on DKPs isolated from *Vibrio* sp. associated with marine sponge *D. avara*. Positive activities: **A** Cyclo-(L-prolyl-L-leucine) [0.5 mg (+/-), 1 mg (+), 2 mg (++)], **B** cyclo-(L-prolyl-L-phenylalanine) [0.5 mg (+), 1 mg (++), 2 mg (+++)].

The dichloromethane extract (216.4 mg) of cell-free supernatant of *Bacillus* sp. (from *I. variabilis*) was purified by Lobar RP-18 column chromatography. Seven compounds were characterized by NMR analysis (^1H, ^{13}C), and identified as cyclo-(*cis*-4-hydroxy-D-prolyl-L-leucine) (**5**), cyclo-(*trans*-4-hydroxy-L-prolyl-L-leucine) (**6**), cyclo-(glycyl-L-leucine) (**7**), cyclo-(*cis*-4-hydroxy-D-prolyl-L-phenylalanine) (**8**), cyclo-(*trans*-4-hydroxy-L-prolyl-L-phenylalanine) (**9**), cyclo-(D-prolyl-L-tyrosine) (**10**) and cyclo-(L-

prolyl-L-tyrosine) (**11**) [10]. Of the seven isolated diketopiperazines, compound **5**, isolated from the active fraction BACF4 (66.7 mg, eluted with H_2O/MeOH 6:4), activated the NTL4 bioreporter (data not shown).

Species-specific cell-cell signalling is involved in pathogenic or symbiotic interactions between a variety of bacteria and their plant and animal hosts [12]. It has been demonstrated that QS molecules are involved in the settlement of the green seaweed *Ulva* onto marine surfaces, and the detection of AHLs results in calcium influx into the zoospore. That was the first example of a calcium signalling event in a eukaryote in response to bacterial QS molecules [13]. Moreover, it has been shown that the interdial surfaces colonized by *Ulva* are dominated by *alpha-proteobacteria*, and that this diverse assemblage both produces and degrades AHLs. These results suggested that AHL-degrading strains can affect bacterial community behavior by interfering with QS between neighboring bacteria [14]. It may, therefore, be the case that some kind of "communication" also exists between marine sponges and microorganisms that are specifically associated with them. A range of AHLs was detected in *alpha-* and *gamma-proteobacteria* isolated from the marine sponges *Mycale laxissima* and *Ircinia strobilina*, and, among the bacteria tested, AHL production was more frequently observed for the *Proteobacteria* associated with *M. laxissima* than those with *I. strobolina* [15].

The beneficial coexistence of microorganisms and sponge is well documented. Multispecies bacterial communities are species-specific and regulate their behavior by intraspecies and interspecies cell-cell communication [16, 17]. The finding of two different bacteria, associated with the same sponge (*D. avara*), with contrasting responses in bioassay for AHL synthesis, could add an interesting dimension to the study of signalling interactions in sponge microcosms. This peculiar behavior could be part of the mutual control on the growth of different microorganisms in the same host.

In a previous paper the isolation and chemical characterization of compounds belonging to the diketopiperazine class from cell-free supernatant of *Bacillus* sp. associated with the marine sponge *I. variabilis* has been described [10]. De Rosa *et al.* [10] proposed a role of cyclo-(*cis*-4-hydroxy-D-prolyl-L-leucine) in the QS mechanism. Now we have demonstrated that this compound, present in both *Bacillus* sp. and *Vibrio* sp. strains, activated AHL bioreporters (CV026 and NTL4) . Moreover other cyclic dipeptides, cyclo-(L-prolyl-L-leucine) and cyclo-(L-prolyl-L-phenylalanine), isolated from *Vibrio* sp. associated with the marine sponge *D. avara* showed the ability to activate QS bioreporters. It is reported that QS is mediated by molecules such as AHL or small peptides. Moreover, other low molecular mass diffusible factors are also described as modulators of communication in bacterial communities [18]. It is not unexpected that from cell-free supernatants of marine sponge-associated bacteria we isolated cyclic dipeptides as factors involved in the QS mechanism. This finding will be useful for studying cross-kingdom chemical communication.

Experimental

Isolation of strains: Samples of *D. avara* and *G. cynodium* were collected in the bay of Naples at a depth of 20-25 m, and kept in fresh seawater during transport to the laboratory. Sponges were rinsed in sterile seawater twice, then transferred into new fresh sterile seawater, and the surface carefully removed. The mesophyll tissues were once more rinsed with sterile seawater and cut into small pieces. These were inoculated in either 20 mL Bactomarine (bactopeptone 5 g L^{-1}; yeast extract 1 g L^{-1} in artificial sea water) or 20 mL Bactomarine supplemented with a pool of antibiotics (ampicillin 100 μg mL^{-1}, kanamycin monosulfate 100 μg mL^{-1}, tylosin tartrate 100 μg mL^{-1}, tetracycline 100 μg mL^{-1}, gentamicin sulfate 10 μg mL^{-1}). After 72 h of incubation at 20°C, a 100 μL aliquot of each culture was spread onto fresh solid Bactomarine. After 72 h incubation at 20°C, single colonies were transferred onto solid fresh medium.

Identification of strains: Isolated strains were identified by PCR analysis. Complete 16S sequences for each bacterial species were obtained as reported by Tommonaro *et al.* [7]. rRNA sequences were submitted to GenBank.

Antibiotic resistance test: The isolated marine bacteria were exposed to the same pool of antibiotics, and at the same concentration, to that used in the isolation step. Briefly, bacteria were grown in 10 mL of Bactomarine supplemented with ampicillin, kanamycin monosulfate, tylosin tartrate, tetracycline (at concentration 100 μg mL^{-1}) and gentamicin sulfate (10 μg mL^{-1}) at 30°C on a rotary shaker overnight. The growth was monitored by O.D. measurement at λ = 540 nm.

Bioassays for putative AHL signal molecules

Lux-screen assay: The presence of putative AHL signal molecules was initially detected using the *E. coli* lux-based biosensors pSB536, pSB401 and pSB1075 [19]. Spent medium (10 L) from stationary phase cultures of all isolated strains was analyzed as described by Tommonaro *et al*. [7].

TLC-overlay: Supernatant extracts were applied to C-18 RP-TLC plates (20 cm x 20 cm; VWR International) and TLC-overlay assay was carried out as described by Tommonaro *et al*. [7]. This test was also performed as spot tests on purified compounds. Different amounts (from 0.5 mg up to 2 mg) of isolated active diketopiperazines were applied to Silica gel TLC plates. Plates were overlaid with 100 mL of LB medium soft agar supplemented with chloramphenicol (30 μg mL^{-1}) and kanamicin (25 μg mL^{-1}) and inoculated with CV026 bioreporter. The TLC plates were kept in a sterile container and incubated at 30°C for 24-48 h.

Plate T-streak: All strains were tested by T-streak assay with *A. tumefaciens* NTL4 biosensor to detect the production of AHL molecules. Because marine bacteria did not grow on ATGN medium (used for biosensor strain), we developed a new method to test the production of AHL molecules. We prepared plates with two solid media, Bactomarine and ATGN, side by side. Marine bacteria were streaked onto Bactomarine and *A. tumefaciens* NTL4 was inoculated into ATGN medium soft agar (0.6%, w/v); plates were then incubated at 30°C for 24-48 h. All strains were also tested by T-streak assay with *C. violaceum* to detect inhibition or degradation of AHL molecules. Both *C. violaceum* and marine bacteria were streaked on the same plate with Luria Bertani (LB) solid medium to form a "T". An inhibition or degrading activity is detected by a visible negative gradient of violacein production.

Isolation and chemical characterization of diketopiperazines from Bacillus sp. and* Vibrio *sp.: The isolation and chemical characterization of cyclic dipeptides were performed as previously described [10]. Briefly, the spent medium (10 L) of *Vibrio* sp. and *Bacillus* sp. was extracted twice with dichloromethane. After drying under vacuum, the dichloromethane extracts were purified by Lobar RP-18 columns eluted with a gradient of H_2O/CH_3OH starting from 9:1 up to pure CH_3OH (with steps of 100 mL); fractions of 100 mL were collected. All recovered fractions were assayed for activation

of QS by TLC-overlay test using *A. tumefaciens* NTL4 and CV026 bioreporters. The active fractions were further chromatographed on a Lobar RP-18 column eluted with a gradient of H_2O/CH_3OH starting from 9:1 up to pure CH_3OH (each step of 100 mL; flow 2 mL/min, fractions of 5 mL were collected). Purified fractions were characterized by NMR (^1H, ^{13}C) analysis with the aim of chemically characterizing active compounds. ^1H NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD_3OD; ^{13}C spectra were recorded on a Bruker AMX-300 spectrometer in CD_3OD.

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