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Evidence for Acyl Homoserine Lactone Signal Production in Bacteria Associated with Marine Sponges

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We report for the first time the production of acyl homoserine lactones (AHLs) by bacteria associated with marine sponges. Given the involvement of AHLs in bacterial colonization of many higher organisms, we speculate that such quorum sensing signals could play a part in interactions between sponges and the dense bacterial communities living within them.

Many gram-negative bacteria utilize acyl homoserine lactone (AHL)-mediated signaling systems to communicate with one another (9, 27, 33). These systems involve the production of low-molecular-weight molecules that accumulate with increasing bacterial numbers and thus provide an index of population density. When a threshold bacterial density (and corresponding AHL concentration) is reached, AHLs interact with transcriptional activators to trigger the expression of target genes. Many terrestrial bacteria produce AHLs, yet, beyond the well-characterized *Vibrio fischeri*-squid symbioses (3, 22, 24), relatively little is known about the occurrence of AHLs in marine environments. The recent finding that bacteria in marine snow are capable of producing AHLs (12) suggests that such signaling molecules could be widespread. In other studies, the addition of AHLs and cyclic AMP (4) or siderophores (14) to marine growth media increased bacterial culturability and growth, respectively.

Many AHL-regulated phenotypes are involved in bacterial colonization of higher organisms, e.g., bioluminescence in *V. fischeri* (22, 24) and conjugal plasmid transfer in *Agrobacterium tumefaciens* (23, 36). Given the dense bacterial communities associated with marine eukaryotes such as sponges (8, 34, 35), macroalgae (18, 20), or ascidians (29, 30) and the density-dependent nature of AHL signaling, we targeted benthic eukaryotes as likely sources of AHL-producing bacteria. Marine sponges in particular are notable for their diverse microbial biota (15, 16, 32), with bacterial densities in some sponges exceeding those in surrounding seawater by 2 to 3 orders of magnitude (7). Here, we demonstrate for the first time the production of AHLs by bacteria isolated from sponges and the apparent in situ production of AHLs within sponge tissue.

Marine organisms, or portions thereof, were collected by scuba diving at depths of 1 to 10 m from several coastal locations near Sydney, in southeastern Australia. Duplicate individuals were sampled for species from the following taxa: sponges (31 species); macroalgae (30 species); and sea grasses, bryozoans, ascidians, and corals (2 species each). Direct screening of small tissue samples by the *Chromobacterium violaceum* CV026 bioassay (21) revealed the presence of AHL-producing bacteria in all host phyla examined (Table 1). While AHLs appeared to be widespread in marine eukaryote-bacterium associations (especially those involving sponges), there was also substantial variability (both over time and within species) in their occurrence. For example, in a separate short-term sampling program, AHLs were detected in only two out of eight sponge species tested on day 1 but were found in all eight species when collected on day 9.

These preliminary investigations using the *C. violaceum* CV026 bioassay demonstrated the utility of this approach for initial screening. However, other methods are required to more adequately describe the occurrence of AHLs in these associations. A role for AHL regulation in any system implies high bacterial densities. Total bacterial cell counts were therefore performed for the sponges *Cymbastela concentrica*, *Siphonochalina* sp., and *Tedania digitata* with DAPI (4′,6′-diamidino-2-phenylindole) staining of fixed, homogenized sponge tissue. Triplicate samples were analyzed for each species of sponge, and 10 fields of view were counted for each sample. Bacterial densities were as follows: for *Cymbastela concentrica*, 3.82×10^8 cells/g (fresh weight) (standard error = 1.69×10^7); for *Siphonochalina* sp., 2.69×10^8 cells/g (4.24×10^7); and for *T. digitata*, 9.85×10^7 cells/g (1.03×10^7). These densities were similar in magnitude to those in other marine sponges (7, 31), much higher (100- to 1,000-fold) than those typical for seawater (6), and comparable to those reported for detectable AHL production (11–13).

AHL-producing bacteria were then isolated from marine sponges by two approaches. The first was a targeted isolation of bacteria that induced violacein production (indicating the presence of AHLs) during tissue screening with *C. violaceum* CV026. Although biased towards the isolation of fast-growing bacteria, this method gives a high probability of successfully culturing at least some AHL producers. This resulted here in the first report of AHL production by the sponge-associated bacterium *Vibrio* sp. (tentatively identified as *Vibrio campbellii*;

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TABLE 1. AHL production in marine invertebrate and plant tissue samples, as assessed by the *C. violaceum* (CV026) bioassay performed in duplicate

Taxon	AHL production (no. of species activating assay/ no. of species tested)
Marine invertebrates	27/37
Sponges	24/31
Corals	1/2
Ascidians	1/2
Bryozoans	1/2
Marine plants	13/32
Macroalgae	
Brown algae	5/14
Green algae	3/5
Red algae	3/11
Sea grasses	2/2

NCBI accession no. X74692; 16S ribosomal DNA [rDNA] sequence similarity, 99.3%). Specific AHLs were identified by running ethyl acetate extracts of bacterial supernatants with selected AHL standards on reverse-phase thin-layer chromatography plates (Merck). Plates were developed in methanol-water (60:40, vol/vol), air dried, and overlaid with the AHL assay organism *A. tumefaciens* A136 (25) or *C. violaceum* CV026 (21). Supernatant extracts were also analyzed using gas chromatography-mass spectrometry (GC-MS) for 3-oxo (5) and non-3-oxo (26) AHLs. Bioassays and GC-MS both demonstrated the production by *Vibrio* sp. of *N*-hexanoyl-homoserine lactone and *N*-(3-oxo)-hexanoyl-homoserine lactone. Another, more traditional approach to isolating AHL producers was also used. Homogenized material from the sponge *Cymbastela concentrica* was spread plated on growth medium, and the predominant colony types were screened for AHL production by streaking adjacent to *C. violaceum* CV026 and *A. tumefaciens* A136. Of 11 isolates tested, one was an AHL producer. 16S rDNA sequencing identified this organism as an α-proteobacterium in the *Roseobacter-Ruegeria* subgroup (NCBI accession no. AB026194; 16S rDNA sequence similarity, 99.4%). Representatives of this taxon found in marine snow also produce AHLs (12).

Screening of tissue samples and isolates can indicate the presence of AHL-producing bacteria within marine eukaryotes but cannot confirm in situ production of AHLs. We therefore screened for AHLs directly in chemical extracts of sponge tissue. Methanol extracts were made for duplicate individuals from each of 11 sponge species (*Callyspongia* sp. 1, *Callyspongia* sp. 2, *Callyspongia* sp. 3, *Ciocalypta* sp., *Cymbastela concentrica*, *Halichondria* sp., *Raspailia* sp., *Siphonochalina* sp., *Spongia* sp., *Stylinos* sp., and *T. digitata*). Crude extracts were initially screened for AHLs by GC-MS and the *A. tumefaciens* A136 and *C. violaceum* CV026 bioassays, and then all extracts were further partitioned into ethyl acetate. The ethyl acetate extracts were then chromatographed on SPE-DPA columns (Supelco) with a series of hexane-ethyl acetate mixtures (95:5, 65:35, 25:75, and 0:100). The resulting four fractions were taken to dryness in vacuo and tested for the presence of AHLs by the previously mentioned assays. A control was also included, in which only the volume of solvent used for the initial extraction was passed through a Discovery DPA 65 solid-phase extraction column.

No evidence of AHLs was found in crude extracts of sponge tissue, by either bioassays or GC-MS. However, purification of crude extracts resulted in activity for several fractions in the *A. tumefaciens* A136 and/or *C. violaceum* CV026 bioassays (Table 2). While some of the hits in the CV026 inhibition assay could be due to growth inhibition by extract components, more solid evidence for the presence of AHLs or AHL-like compounds was seen in the positive responses with both bioassays from four extracts and a positive response in *A. tumefaciens* A136 only from two extracts (Table 2). Furthermore, most activity was detected in the second fraction of the crude extracts, which corresponds to elution of selected AHL standards from a separate SPE-DPA column (data not shown). AHLs were not detected in the extracts with the use of GC-MS; however, in a separate study (P. J. Schupp et al., unpublished data) this method was found to be up to 50-fold less sensitive with environmental samples than was the *A. tumefaciens* A136 bioassay.

Other putative signal molecules, including diketopiperazines (DKPs), interact with AHL systems (17) and have been isolated from marine sponges (1, 19). However, we found no evidence of DKPs when screening sponge extracts using GC-MS (data not shown). Given the relatively high concentrations of DKPs required to activate AHL bioassays (17), we consider it unlikely that these molecules were responsible for the hits in our assay. Other molecules that mimic AHLs—and in some cases inhibit AHL phenotypes (10)—have also been reported (2, 28), and such compounds may occur in sponge-bacterium associations.

To our knowledge, this is the first report of AHL production by bacteria associated with marine sponges. Another, previous study was unable to demonstrate AHL production by bacteria from two tropical sponges (14). In addition, our data suggest that AHLs (or molecules capable of upregulating AHL biosensors) are being produced in situ within sponges. The finding of such signals in marine sponge-bacterium associations is consistent with both the very high bacterial densities present in these hosts and the role of AHLs in bacterial colonization of higher organisms. Identification of the phenotype(s) under AHL regulation is a crucial next step. We speculate that AHLs could provide one mechanism by which the intimate associations between marine sponges and microbes are established and maintained.

TABLE 2. Activities of partially purified methanolic extracts from 11 marine sponges in *A. tumefaciens* A136 and *C. violaceum* CV026 bioassays[a]

Sponge	A136 assay	CV026 (inhibition) assay
Callyspongia sp. 1	++	−−
Callyspongia sp. 2	−−	++
Callyspongia sp. 3	++	++
Ciocalypta sp.	−−	++
Cymbastela concentrica	−−	++
Halichondria sp.	++	++
Raspailia sp.	−−	++
Siphonochalina sp.	++	++
Spongia sp.	++	−−
Stylinos sp.	−−	++
Tedania digitata	++	++

[a] Results are shown as presence (++) or absence (−) of signal for duplicate samples per species of sponge.

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