

# Antagonistic interactions of sponge-associated Actinobacteria against heterotrophic bacteria from sponge and ambient water

N. S. Subina<sup>1</sup>, C. S. Sonam<sup>2</sup>, Shanta Nair<sup>1</sup> and Maria-Judith Gonsalves<sup>1,\*</sup>

<sup>1</sup>Aquatic Geomicrobiology Laboratory, CSIR-National Institute of Oceanography, Goa 403 004, India

<sup>2</sup>School of Biotechnology, Amrita Vishwa Vidyapeetham, Kollam 690 525, India

**In the present study, 11 sponge-associated Actinobacteria showed 316 inhibitory interactions against 75 heterotrophic bacteria as each Actinobacteria inhibited the growth of >1 bacterial isolates from the sponge and ambient water. The antagonistic activity depended on the source of isolation and the taxonomic group of heterotrophic bacteria. The order of inhibition of bacteria by Actinobacteria was ambient water > cortex > mesohyl tissues of sponge. The absence of certain genera in the sponge might be because of the inhibition by sponge-associated Actinobacteria. Hence, the antagonistic property of Actinobacteria in the sponges may influence the selection of resident sponge-associated bacteria.**

**Keywords:** Actinobacteria, antagonistic activity, heterotrophic bacteria, sponge.

SPONGES are important components of benthic invertebrates and one of the richest sources of secondary metabolites from the marine environment<sup>1</sup>. They harbour numerous microorganisms in their body with bacteria constituting up to 40% of their body mass<sup>2</sup>. Microbes play a fundamental role in driving the chemical cycling processes that ultimately control the health and ecology of sponges<sup>3</sup>. These associated bacteria also play an important role in terms of mechanical support of the sponges, elimination of waste products, autotrophy, bioluminescence, nitrogen metabolism and/or protection from UV radiation<sup>4-6</sup>. Sponges form three types of bacterial associations, viz. 'sponge specialists', found only in one host species; 'sponge associates' found in multiple hosts, but not in sea water, and 'generalists' found in multiple hosts and sea water<sup>6</sup>. As sponges are filter feeders, they filter a large number of planktonic bacteria. However, most bacteria in the sponges (both sponge specialists and sponge associates) are phylogenetically diverse and different from those in ambient water<sup>3-5,7</sup>. This variation in the type of microbial association reveals that apart from the physiological condition/nature of the host, the bacteria-bacteria interaction may structure the bacterial community in the sponges.

Actinobacteria are prolific producers of antibiotics<sup>8</sup>. Culture-independent studies showed the existence of Actinobacteria in several sponge species<sup>3,9-12</sup>. However, sponge-associated actinobacterial studies are limited<sup>11</sup> and are often restricted to novel antibacterial compounds against pathogenic bacteria and/or industrial applications<sup>11,12</sup>. More so, the ecological implications of sponge-associated Actinobacteria have not been studied. We hypothesized that Actinobacteria associated with sponges may contribute to the structuring of the sponge-associated heterotrophic bacterial community. Hence, we tested the ability of Actinobacteria associated with the sponges to inhibit the growth of bacteria from ambient water as well as from a sponge.

## Materials and methods

### *Sample collection and processing*

Sponge, *Cinachyrella cavernosa* (class: Demospongiae; order: Spirophorida and family: Tetillidae) and ambient water from rock pool were collected from the rocky intertidal region of Anjuna beach, Goa, India (15°34'0"N, 73°44'0"E) during low tide. The sponge samples were removed carefully and washed with filtered and autoclaved sea water till they were visibly clean of any debris and sediment. Cortex and mesohyl tissues were aseptically separated using a sterile scalpel and homogenized using sterilized mortar and pestle in artificial sea water (1.1 g CaCl<sub>2</sub>, 10.2 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 31.6 g NaCl, 0.75 g KCl, 1.0 g Na<sub>2</sub>SO<sub>4</sub>, 2.4 g Tris-HCl, 0.02 g NaHCO<sub>3</sub>, 1 l distilled water, pH 8.1) (1 : 9 weight/volume)<sup>13</sup>. The homogenate was serially diluted for enumeration and isolation of Actinobacteria and heterotrophic bacteria (HB). The latter were also isolated from sponge ambient water.

### *Enumeration and isolation of cultivable Actinobacteria and heterotrophic bacteria*

Different selective media such as marine sponge agar (MSA), casein starch agar (CSA) and inorganic salt

\*For correspondence. (e-mail: mjjudith@nio.org)

starch agar (ISP-4) supplemented with 25 µg ml<sup>-1</sup> cycloheximide to control fungal growth and 25 µg ml<sup>-1</sup> nalidixic acid to inhibit Gram-negative bacteria were used for the retrieval of a maximum number of Actinobacteria isolates<sup>14</sup>. Sponge homogenate (100 µl from 10<sup>-1</sup> to 10<sup>-3</sup> dilution) was spread-plated on the above-mentioned media in triplicate and incubated at 25° ± 2°C for 2–3 weeks. For HB, the sponge homogenate (10<sup>-1</sup>–10<sup>-5</sup> dilution) and ambient water (10<sup>0</sup>–10<sup>-2</sup> dilution) were surface-plated in triplicate on Zobell Marine Agar (ZMA) and incubated at 25° ± 2°C for 48 h. Bacterial colonies were counted and abundance is expressed as colony forming units (CFU) cm<sup>-3</sup>. Twenty-four morphologically different Actinobacteria colonies were isolated and based on the incubation time for growth (<2 weeks) on the sub-culturing, 11 Actinobacteria (from mesohyl (MAb 1-7) and cortex (CAB1-4) tissues of sponge) were selected for antagonistic assay. Seventy-five morphologically and biochemically different HB were isolated from the sponge and ambient water (M01-25, C01-25 and W01-25 from mesohyl and cortex tissues of sponge and ambient water respectively; [Supplementary Table 1](#)), and were used for antagonistic assay. The purified isolates of Actinobacteria and HB were identified using PIBWIN software, based on *Bergey's Manual of Determinative Bacteriology*. The tests used for identification of Actinobacteria were colony characteristics, spore chain and cell morphology, Gram's reaction, growth in sodium azide, growth in lysozyme, growth at different values of pH and temperature, enzyme production, carbon utilization, antibiotic susceptibility, nitrate reduction, hydrogen sulphide production and tyrosine degradation. The tests used for HB identification were colony characteristics, cell morphology, Gram's reaction, catalase, oxidase, oxidative/fermentative test (OF test), motility, IMVIC test, carbon utilization, enzyme production, nitrate reduction and growth on MacConkey agar.

### Antagonistic study

**Preparation of Actinobacteria:** Actinobacteria was inoculated into ISP-4 broth and incubated at 25° ± 2°C for two weeks to attain maximum growth. The cells were removed by centrifugation at 2000 g for 20 min. The supernatant obtained was filtered through 0.2 µm filter to remove the spores. The filtrate was used for antagonistic study against HB isolates.

**Preparation of HB suspension:** Heterotrophic bacteria was grown in Muller–Hinton broth and incubated at 25° ± 2°C for 12–18 h. The bacterial cells were pelleted by centrifugation at 8000 g for 10 min. The pellet was re-suspended in sterile saline solution and the bacterial density was adjusted to 10<sup>6</sup> cells ml<sup>-1</sup> by comparing with McFarland standard.

**Antagonistic assay:** The antagonistic assay was performed according to Sarker *et al.*<sup>15</sup>, with modification. The assay used resazurin as growth indicator<sup>16</sup>. If bacterial growth occurs, this blue dye becomes pink when reduced to resorufin by oxidoreductases. It is further reduced to colourless hydroresorufin. For the assay, 50 µl Actinobacteria filtrate and 10 µl resazurin (6.75 mg ml<sup>-1</sup>) solution were added to the wells of a microtitre plate. To this, 30 µl Muller–Hinton broth was added followed by 10 µl of HB suspension to reach a final bacterial density of 10<sup>5</sup> cells ml<sup>-1</sup>. Three sets of control were used, viz. (1) broad spectrum antibiotic ciprofloxacin, (2) without Actinobacteria filtrate, and (3) without HB in the wells of the microtitre plate. Plates were prepared in triplicate and wrapped with cling film to ensure that bacteria do not get dehydrated. The experimental plates were incubated at 25° ± 2°C for 48 h. Any colour change from blue to pink or colourless indicated growth of inoculated HB. No change in blue colour indicated inhibition of growth of HB by Actinobacteria.

**Statistical analysis:** The difference in the abundance of HB among different sources was tested by one-way ANOVA. The antagonistic potential of cortex and mesohyl tissues of the sponge was calculated as the ratio of the number of positive antagonistic interactions by Actinobacteria and their total number in cortex and mesohyl respectively. The significance of the difference in antagonistic potential of different sources was checked using *t*-test (Statistica 6). In order to visualize the antagonistic interactions, the network was created using Cytoscape. One-way analysis of similarity (ANOSIM) was used (Primer-6) to find out the significance of difference in the inhibition between different HB sources.

## Results and discussion

### Abundance of Actinobacteria in sponge

Culture-independent studies have shown that Actinobacteria are an important component of the microbial assemblage of sponges<sup>5,9,17</sup> and harbour antagonistic genes code for enzymes like non-ribosomal synthase and polyketide synthase<sup>14</sup>. The number of Actinobacteria present in the mesohyl and cortex tissues of the sponge ranged from 10<sup>2</sup> to 10<sup>4</sup> CFU cm<sup>-3</sup> (1.9 × 10<sup>2</sup>–1.3 × 10<sup>4</sup> CFU cm<sup>-3</sup> and 1.7 × 10<sup>2</sup>–2.0 × 10<sup>4</sup> CFU cm<sup>-3</sup> in mesohyl and cortex respectively). The growth of Actinobacteria depends on species, medium and source. The abundance of Actinobacteria was different in different sections of the sponge. CSA showed the maximum number of Actinobacteria from cortex and ISP-4 showed maximum number from mesohyl tissues (Figure 1a). Minimum number of Actinobacteria from both cortex and mesohyl was obtained in MSA. Such variation in retrieval

counts using different media has been reported earlier where the authors<sup>14</sup> used whole sponge tissue for the enumeration of Actinobacteria in different media and found a higher abundance of Actinobacteria associated with the sponge, *Dendrilla nigra* in MSA than CSA. A total of 11 different morphotypes of Actinobacteria belonging to Actinoplanaceae and Streptomycetaceae families were isolated from the sponge (Table 1). The characteristics of 11 Actinobacteria used for identification are given in the [Supplementary Table 2](#). Several actinobacterial selective media have been formulated for their maximum retrieval. However, culturing marine Actinobacteria is difficult compared to its terrestrial counterparts, due to the salinity requirement. Among marine bacteria, the longer incubation time for Actinobacteria than heterotrophic bacteria makes actinobacterial culturing difficult<sup>18</sup>. *Actinoplanes* and *Streptomyces* were the common genera present in the mesohyl and cortex tissues of the sponge. The presence of *Streptomyces* sp. has been reported in sponges<sup>14,19</sup>. It was reported that *Streptomyces* is the dominant producer of antibiotics among the different actinobacterial genera<sup>20,21</sup>. The dominance of *Streptomyces* sp. in sponges may be due to its ability to inhibit a multitude of fast-growing bacteria from its own habitat, thereby avoiding competition for utilizing the nutrients available in its niche<sup>20,21</sup>.

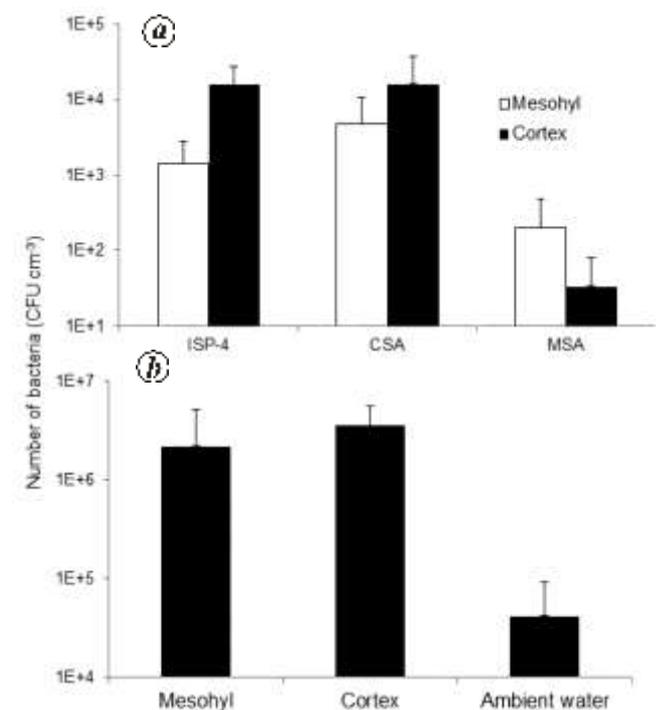
#### Abundance of heterotrophic bacteria in sponge and ambient water

Culturable HB abundance in the tissues of sponge and in ambient water showed significant difference ( $P < 0.05$ ). The number of HB in the sponge ( $10^5$  to  $10^6$  CFU  $\text{cm}^{-3}$ ) was two orders higher than that in ambient water ( $10^3$  to  $10^4$  CFU  $\text{cm}^{-3}$ ) (Figure 1 b). This greater abundance of bacteria in the sponge than ambient water might be due to the favourable physiological conditions and accessibility of nutrients in the former for bacterial growth<sup>14</sup>. High bacterial abundance was observed in sponge cortex, which was 2–5 times higher than that of mesohyl tissues of sponge, as this intertidal sponge is always covered with sediments which allow sediment-derived bacteria to invade to the cortex of the sponge<sup>7</sup>. Also, marine bacterioplanktons have natural tendency to form biofilm (epibiosis) on living or non-living substances<sup>22</sup>. For example, marine *Alteromonas*, *Bacillus*, *Chromobacterium*, *Pseudomonas*, *Serratia* and *Vibrio* were reported to be fouling organisms<sup>23–25</sup>.

#### Actinobacteria–heterotrophic bacterial interaction

The antagonistic potential of sponge-associated Actinobacteria against a wide range of organisms such as bacteria, fungi and parasites has been reported<sup>19,26,27</sup>. Most of the studies have examined the antagonism using primarily

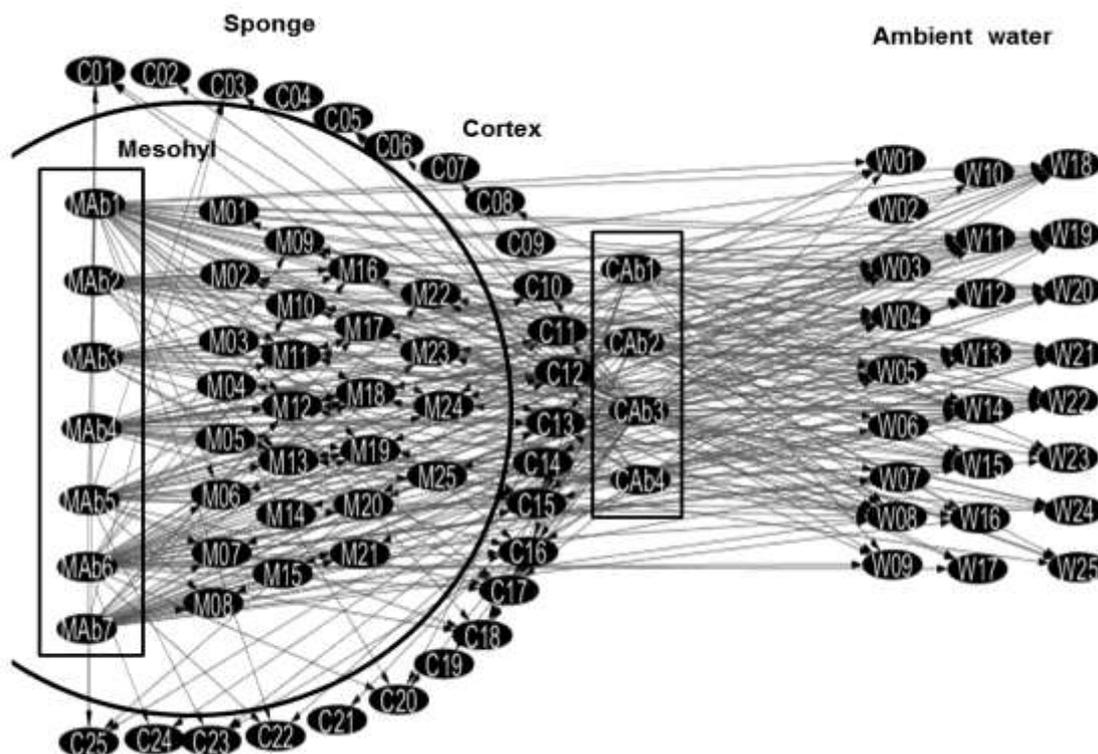
pathogens and have found up to 20% of sponge isolates to be inhibitory<sup>28</sup>. We studied the antagonistic interactions of Actinobacteria against 75 HB from the same sponge and ambient water using  $11 \times 75$  array of tests. Eight hundred and twenty-five tests showed that all Actinobacteria associated with sponge inhibited the growth of more than one HB. A total of 316 antagonistic interactions were exhibited by Actinobacteria, among which 157 interactions (49.7%) were against HB from ambient water (Figure 2, [Supplementary Table 3](#)). ANOSIM showed that antagonistic interactions of Actinobacteria with HB from the ambient water were significantly higher than those with sponge-associated HB



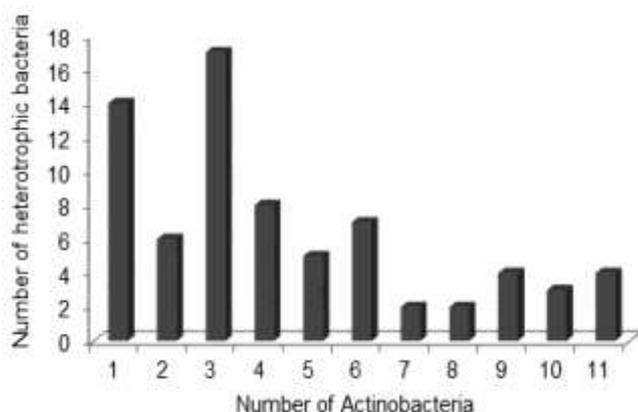
**Figure 1.** a, Abundance of Actinobacteria from mesohyl and cortex tissues of sponge in different selective media. b, Abundance of heterotrophic bacteria in mesohyl and cortex tissues of sponge and ambient water.

**Table 1.** Actinobacteria in the mesohyl and cortex tissues of sponge

Sponge	Isolate	Actinobacteria
Mesohyl tissue	MAb1	<i>Actinoplanes consettensis</i>
	MAb2	<i>Streptoverticillum olevoreticuli</i>
	MAb3	<i>Streptoverticillum olevoreticuli</i>
	MAb4	<i>Streptomyces thermovulgaris</i>
	MAb5	<i>Actinoplanes careuleus</i>
	MAb6	<i>Streptomyces cyaneus</i>
	MAb7	<i>Streptomyces thermovulgaris</i>
Cortex tissue	CAb1	<i>Streptomyces prasinosporus</i>
	CAb2	<i>Streptomyces sxaanthochromogens</i>
	CAb3	<i>Streptomyces thermovulgaris</i>
	CAb4	<i>Actinoplanes caeruleus</i>



**Figure 2.** A network of 316 antagonistic interactions by mesohyl and cortex Actinobacteria (MAb and CAb respectively) against HB from sponge mesohyl (M), cortex (C) and ambient water (W), generated using Cytoscape. Nodes represent bacterial isolates (Actinobacteria and HB) and directed edges represent the antagonistic relationship of the isolates.



**Figure 3.** Multiple inhibitions of Actinobacteria against HB.

(Global  $R = 0.03$ ,  $P < 0.05$ ) and against different bacterial genera (Global  $R = 0.132$ ,  $P < 0.001$ ). Multiple inhibitions or multiple antagonisms (inhibition of  $\geq 2$  HB) were also observed among Actinobacteria. More than one Actinobacteria inhibited 58 HB (Figure 3). The order of inhibition of bacteria based on the source of isolation was ambient water > cortex tissue > mesohyl tissue of sponge. Eleven HB (44%) from the ambient water exhibited multiple inhibitions by >7 Actinobacteria. This might be the reason for the lower bacterial diversity in the sponge<sup>8,29,30</sup> in spite of filtering about 16 l of ambient water per day

(Dahihande and Thakur, pers. commun.), and higher bacterial abundance in sponge than ambient water<sup>7,29,30</sup>. The overall antagonistic potential of the sponge was 28.7, and antagonistic potential of the cortex tissue was higher than that of the mesohyl tissue (35.0 and 25.1 respectively). Intra-antagonistic interaction showed that antagonistic potential of cortex Actinobacteria against cortex HB was 10.3, and mesohyl Actinobacteria against mesohyl HB was 5.3. Antagonistic activity can confer competitive advantage within a bacterial community by keeping sensitive isolates<sup>31</sup>.

CAb3 (*Streptomyces thermovulgaris*), isolated from the sponge cortex showed the highest number (68) of inhibitions (inhibition of 23, 22 and 23 HB from ambient water, cortex and mesohyl tissues respectively), followed by MAb6 (*Streptomyces cyaneus*) isolated from the mesohyl tissue (Figure 4). Bhattarai *et al.*<sup>32</sup> found that 50% of the marine antagonistic strains belonged to Actinobacteria. This high interaction of Actinobacteria might be because of their inherent potential to produce several antimicrobial substances which keep other bacteria away from their niche<sup>19,33</sup>. A minimum number of inhibitions was observed by MAb2 and Mab3 (*Streptovorticillum olevoreticuli*) isolated from the mesohyl tissue of the sponge, which inhibited >50% HB from ambient water.

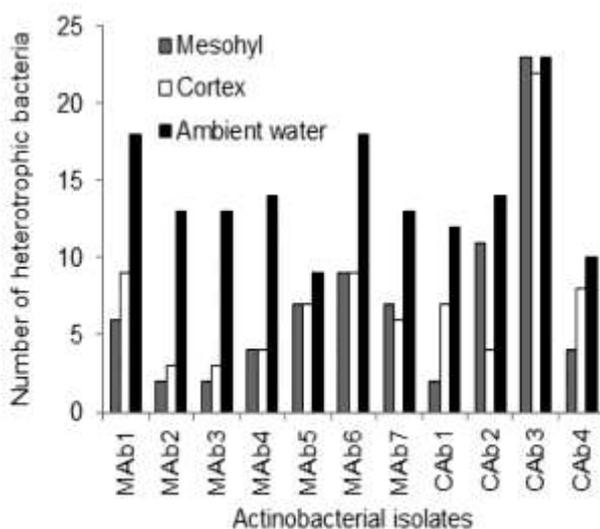
We found that the antagonistic activity of sponge-associated Actinobacteria was dependent on the source of

isolation as well as their taxonomic group. Mangano *et al.*<sup>34</sup> found that sponge-associated Actinobacteria tend to inhibit species of the same taxonomic group. The results of our study suggest that host sponge may possibly exert some selective pressure on the associated bacteria, apart from habitat structure, animal–host interaction and local biogeochemical conditions. As a consequence, the bacteria associated with a sponge may undergo some genetic changes in order to survive in the sponge environment, which may result in the variation of gene sequences compared to that of their counterparts in the water<sup>35</sup>. For instance, *Brahmella* sp., *Pseudomonas diminuta*, *Vibrio diazotrophicus*, *Vibrio metschnikovii*, *Vibrio nereis* and *Xanthomonas* sp. found in the sponge as well as in ambient water were inhibited by different numbers of AB, depending on their source. The inhibition of these HB by Actinobacteria based on the source of isolation was in the order of ambient water > cortex > mesohyl. Similarly, sponge-associated *Pseudomonas stutzeri* was inhibited by a higher number of Actinobacteria from cortex tissues than from mesohyl Actinobacteria. The differential antagonistic activity of sponge-associated Actinobacteria against different HB might be the reason for the absence of certain genera in mesohyl or cortex tissues of the sponge. For example, *Aeromonas salmonicida* and *Agrobacterium* sp., which were isolated exclusively from mesohyl, were inhibited by Actinobacteria from cortex tissues and hence could not be detected in the cortex. Similarly, *Bacillus badius* was found only in cortex tissue and was inhibited by Actinobacteria from mesohyl. *Acinetobacter* sp. and *Pseudomonas paucimobilis* isolated from mesohyl were inhibited by cortex Actinobacteria; the strains belonging to these species isolated from ambient water were inhibited by both mesohyl and cortex Actinobacteria, and hence could not be detected in cortex

tissues. Similarly, *Flavobacterium* sp. isolated from cortex was inhibited by mesohyl Actinobacteria, and *Flavobacterium* sp. isolated from ambient water was inhibited by mesohyl and cortex Actinobacteria. The results suggest that antagonistic activity of Actinobacteria associated with the sponge may provide an effective control mechanism of microbial populations in the intertidal sponge, *C. cavernosa*. Mangano *et al.*<sup>34</sup> found that the sponge, *Lissodendoryx nobilis* associated bacterial community was more antagonistic against strains isolated from the same sponge species rather than against isolates from different sponge species. The reason might be conferring a selective advantage in the competition for nutrients in *L. nobilis* from oligotrophic Antarctic waters. The strains of *Moraxella* sp. and *Photobacterium* sp. isolated from ambient water were inhibited by a higher number of Actinobacteria than the strains from cortex tissues. It was observed that Actinobacteria from both mesohyl and cortex tissues inhibited *Chromobacterium*, *Erwinia* and *Serratia*, and hence these bacteria could not be detected in the sponge tissues. Hoffman *et al.*<sup>35</sup> suggested that sponge environment imposes strong selection on resident microflora, generally prohibiting habitation by planktonic strains. The present study showed that Actinobacteria associated with sponge inhibited ~50% of the bacterial isolates from ambient water.

The composition and function of bacterial communities associated with sponges are variable and different from those of ambient water. The present study showed that higher number of heterotrophic bacteria from ambient water than sponge-associated bacteria was inhibited by Actinobacteria. Also, the dominant bacteria like *Vibrio* were least inhibited by Actinobacteria. Moreover, some bacterial genera were found in water, but not in the sponge. Hence it has been suggested that heterotrophic bacteria associated with a sponge are likely to be shaped by Actinobacteria by inhibiting bacteria from ambient water to enter the body of the sponge and to selectively inhibit certain groups of bacteria inside the sponge tissue. Further studies on the mechanism of inhibition will unravel the spatio-temporal dynamics of sponge–microbial communities and reveal whether it is a common phenomenon among other sponges. The analysis of antagonistic interactions reported here would serve as a platform for the generation of testable hypothesis on the actual *in vivo* relationships among associated bacterial communities.

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



**Figure 4.** Percentage of HB inhibition by different morphotypes of Actinobacteria.

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ACKNOWLEDGEMENTS. We thank the Director, CSIR-National Institute of Oceanography, Goa for providing the necessary facilities. We also thank the Ministry of Earth Sciences, Government of India for financial support. This is NIO contribution no. 6130.

Received 23 June 2016; revised accepted 6 July 2017

doi: 10.18520/cs/v113/i12/2311-2316