

# Culturable heterotrophic bacteria from the marine sponge *Dendrilla nigra*: isolation and phylogenetic diversity of actinobacteria

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**Abstract** Culturable heterotrophic bacterial composition of marine sponge *Dendrilla nigra* was analysed using different enrichments. Five media compositions including without enrichment (control), enriched with sponge extract, with growth regulator (antibiotics), with autoinducers, and complete enrichment containing sponge extract, antibiotics, and autoinducers were developed. DNA hybridization assay was performed to explore host specific bacteria and ecotypes of culturable sponge-associated bacteria. Enrichment with selective inducers (AHLs and sponge extract) and regulators (antibiotics) considerably enhanced the cultivation potential of sponge-associated bacteria. It was found that *Marinobacter* (MSI032), *Micromonospora* (MSI033), *Streptomyces* (MSI051), and *Pseudomonas* (MSI057) were sponge-associated obligate symbionts. The present findings envisaged that “*Micromonospora–Saccharomonospora–Streptomyces*” group was the major culturable actinobacteria in the marine sponge *D. nigra*. The DNA hybridization assay was a reliable method for the analysis of culturable bacterial community in marine sponges. Based on the culturable community structure, the sponge-associated bacteria can be grouped (ecotypes) as general symbionts, specific symbionts, habitat flora, and antagonists.

**Keywords** Sponge bacteria · Culturable bacteria · Bacterial endosymbionts · *Dendrilla nigra* · Actinobacteria

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## Introduction

Most of the marine invertebrates harbor microorganisms that include bacteria, cyanobacteria, and fungi within their tissues where they reside in the extra- and intracellular space (Lafi et al. 2005; Vacelet and Donadey 1977; Wilkinson 1978; Wilkinson et al. 1981). The presence of large amounts of microorganisms within the mesohyl of many demosponges has been well documented (Hentschel et al. 2002; Imhoff and Stöhr 2003). Sponge mesohyl was referred as “micro-environments” providing a broad variety of ecological niches (Thiel et al. 2007). Bacteria can contribute up to 40% of the sponge biomass (equal to about  $10^8$  to  $10^9$  bacteria  $\text{g tissue}^{-1}$ ) and are probably permanently associated with the host sponge unless they are disturbed by external stress factors (Friedrich et al. 2001; Thoms et al. 2003; Webster and Hill 2001). Sponges are filter feeders and consume microorganisms from the inhaled seawater by phagocytosis. The relationships of marine invertebrates and microorganisms that may serve as food or that lives either permanently or temporarily inside of marine macroorganisms are highly complex and far from being understood (Steinert et al. 2000). The sponge–symbiont relationship could be categorized as obligatory mutualism (i.e., the symbionts play an essential role in the metabolism of their host), facultatively mutualism (they have a beneficial effect on their host, but the host will survive without the symbiont), or commensalism (they are present without providing obvious beneficial effects to their host). In all cases, it is assumed that the sponge host provides a sheltered habitat for their symbionts. A further distinction is made between *epibionts* (microorganisms living on the sponge surface) and *endosymbionts* (microorganisms that either live in the sponge mesohyl or inside the sponge cells) (Osinga et al. 1998). Microorganisms not only serve as food for filter

feeders but may, perhaps, also be involved in the biosynthesis of secondary metabolites that are used for the discovery of novel drugs (Abrell 1997; Debitus et al. 1998; Perovic et al. 1998). In this background, the present study was initiated to explore the culturable heterotrophic bacterial composition of marine sponge *Dendrilla nigra*, which showed broad spectrum of bioactivity (Selvin and Lipton 2004a, b). In this study, we report phylogenetic analysis of cultured actinobacteria and DNA hybridization assay to target sponge-specific bacteria among the most common groups.

## Materials and methods

### Collection of *D. nigra*

The host sponge *D. nigra* was collected from southwest coast of India by SCUBA diving at 12 m depth. The collection site, Vizhinjam coast, is located at 8021'N and 7700'E on the west coast of India comprising of sandy beaches and irregularly distributed intertidal rocky substratum. The temperature of seawater was 25°C. Morphological observation of collected specimens indicated that a unique macrosymbiont, Indian brittle star *Amphiura carchara*, was invariably associated with all the specimens. Therefore, brittle star was separated from the host sponge, enclosed in separate sterile bags, and then transferred to the laboratory on ice. Only unbroken samples were used for microbiological analysis to avoid cross contamination. The specimens kept for 2 h in sterilized aged seawater to remove loosely associated microorganisms from inner and outer sponge surfaces. It has been hypothesized that this process may eliminate non-associated bacteria from the host sponge by digestion. Environmental water representing the sponge habitat was taken prior to sponge sampling and filled up in 1 l sterilized glass bottles. The preserved (frozen) specimens, photographs, and videograph of the collection process and habitat were retained in the laboratory for future reference. Specimens were identified and/or confirmed with the help of renowned sponge taxonomist P.A. Thomas, Scientist Emeritus, Central Marine Fisheries Research Institute, India.

### Isolation and culture of sponge-associated bacteria

One cubic centimeter of sponge tissue was excised from the middle of the whole sponge using a sterile scissors. The excised portion was washed three times with sterile seawater to remove any bacteria within current canals and then the tissue was homogenated using a tissue homogenizer (Omni). The resultant homogenate was serially diluted with sterilized aged seawater and preincubated at 40°C for 1 h for the activation of dormant and inactive cells. The resultant aliquot was plated on various isolation media and

incubated at 25°C in dark aerobic conditions except the anaerobic agar plates until visible colonies appeared. In addition to marine sponge agar (MSA) (Selvin et al. 2004), standard media such as modified marine agar (MMA), seawater agar (SA), modified nutrient agar (MNA), halophilic agar (HA), actinomycetes agar (AA), starch casein agar (CSA), raffinose-histidine medium (RH), fluid-thioglycollate medium (FT), malt agar (MA), Emerson Agar (EA), TCBS Agar (TA), Pseudomonas agar (PA), and anaerobic agar (ANA). The media composition was rationalized and used for the isolation of sponge-associated bacteria. The medium composition per liter of MSA was raffinose 1.0 g, L-histidine 1.0 g, FeSO<sub>4</sub> · 7H<sub>2</sub>O 0.01 g, K<sub>2</sub>HPO<sub>4</sub> 1.0 g, CaCO<sub>3</sub> 0.02 g, MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.5 g, agar 15 g, and NaCl 20 g. The medium was enriched with 10% each of aqueous and organic host sponge extracts, respectively. The ingredients per liter included in the modified media are given below:

- MMA Peptic digest of animal tissue 0.5 g, yeast extract 0.1 g, ferric citrate 0.1 g, sodium chloride 20 g, magnesium chloride 0.8 g, sodium sulfate 0.32 g, calcium chloride 0.18 g, potassium chloride 0.5 g, sodium bicarbonate 0.2 g, potassium bromide 0.08 g, strontium chloride 0.03 g, boric acid 0.02 g, sodium silicate 0.004 g, sodium fluoride 0.002 g, ammonium nitrate 0.002 g, disodium phosphate 0.008 g, sponge organic extract—1%, and dextrose 0.05 g.
- MNA Peptic digest of animal tissue 0.5 g, yeast extract 0.15 g, beef extract 0.15 g, magnesium chloride 0.88 g, dextrose—0.05 g and sodium chloride 20 g.
- SA The composition as in MNA except sodium chloride and the composition was prepared in 1 l aged seawater instead of double distilled water.
- HA NaCl 250.0 g, MgSO<sub>4</sub> · 7H<sub>2</sub>O 2.5 g, casamino acids 1.0 g, yeast extract 1.0 g, protease peptone 0.5 g, trisodium citrate 0.3 g, and KCl 2.0 g.
- AA Beef heart infusion solids 1.0 g, tryptose 1.0 g, casein enzymic hydrolysate 0.4 g, yeast extract 0.5 g, dextrose 0.5 g, L-cysteine hydrochloride 0.1 g, starch 0.1 g, sodium chloride 20.0 g, monopotassium phosphate 1.5 g, ammonium sulfate 1.0 g, magnesium sulfate 0.2 g, and calcium chloride 1.0 g.
- CSA Casein 1.0 g, starch 1.0 g, and seawater 37.0 g.
- RH Raffinose 1.0 g, L-histidine 1.0 g, MgSO<sub>4</sub> 0.5, FeSO<sub>4</sub> 0.01 g, and NaCl 20 g.
- FT Casein enzymic hydrolysate 1.5 g, yeast extract 0.5 g, dextrose 0.5 g, sodium chloride 20 g, L-cystine 0.5 g, sodium thioglycollate 0.5 g, and resazurin sodium 0.001 g.
- MA Malt extract 2.0 g, dextrose 0.5 g, and NaCl 20 g.

- EA Peptic digest of animal tissue 0.4 g, beef extract 0.4 g, yeast extract 1.0 g, dextrose 0.1 g, and sodium chloride 20 g.
- TA Peptone 1.5 g, yeast extract 0.6 g, sodium thiosulphate 1.0 g, sodium citrate 1.0 g, synthetic detergent II 0.2 g, sucrose 2.0 g, sodium chloride 20 g, ferric citrate 1.0 g, bromo thymol blue 0.04 g, and thymol blue 0.04 g.
- PA Casein enzymic hydrolysate 1.0 g, proteose peptone 1.0 g, dipotassium phosphate 0.15 g, magnesium sulfate 0.15 g, and sodium chloride 20 g.
- ANA Casein enzymic hydrolysate 1.2 g, peptic digest of animal tissue 0.5 g, yeast extract 0.3 g, beef extract 0.3 g, corn starch 0.1 g, dextrose 0.1 g, sodium chloride 20.0 g, dithiothreitol 0.1 g, L-cystine hydrochloride 0.5 g, vitamin K1 0.01 g, sponge organic extract 1% and dextrose 0.05 g. The media prepared in 1-l double distilled water and agar 20 g added to obtain solid media.

Another batch of these media were enriched with selective inducers/regulator containing aqueous host sponge extract 2% (sponge agar exempted), organic extract 1%, cAMP (Sigma) 1  $\mu$ M, alpha-butyrolactone 0.1  $\mu$ M, nalidixic acid 10  $\mu$ M, NaCl-2%, and Nystatin 10  $\mu$ M (pH 7.8). The media ingredients were sterilized separately at 121°C, 15 lbs for 10 min, and the selective enrichments were filtered through 0.2  $\mu$  filter (Millipore®) and added to the sterilized ingredients. The host sponge extract was used as “enhancers” of sponge-specific bacterial cultivation potential, the antibiotics, nalidixic acid, and nystatin screened selectively against the surrounding seawater bacteria and fungi, respectively, was used as “regulator” of fast growing bacteria and alpha-butyrolactone was used as “autoinducer” which increase the cultivation potential of unculturable bacteria. Using different enrichments, five media compositions were obtained including: (1) media without enrichment (control), (2) enriched with sponge extract, (3) enriched with nystatin, (4) enriched with alpha-butyrolactone, and (5) complete enrichment containing sponge extract, nystatin, and alpha-butyrolactone. One gram tissue of brittle star was processed in the same way as described for the host sponge. Habitat water samples were serially diluted in sterile-aged seawater and plated for the analysis of habitat microflora. Growth of associated bacteria and free living bacteria in the habitat water was determined in terms of colony forming units (CFU). Only consistent morphotypes in subsequent subcultures were considered, and representatives of the most abundant morphotypes were chosen for DNA hybridization assay. Morphological biochemical and physiological characteristics of bacterial isolates were determined as per standard protocols (Cappuccino and Sherman, 2004; MacFaddin 1981). The

test characteristics were selected on the basis of appropriate previous step of the identification scheme. The antagonistic activity was determined by cross-streaking followed by Burkholder agar diffusion assay (Burkholder et al. 1966).

#### Isolation of DNA and sequencing

Genomic DNA of bacterial symbionts and freeze-dried tissue of host sponge were extracted by the methods as described in earlier reports (Enticknap et al. 2006; Lopez et al. 1999; Pitcher et al. 1989). Both universal and genus-specific primers were used for the amplification of DNA. The reaction mixture was prepared in a total volume of 50  $\mu$ l including 100 ng of template DNA, 5  $\mu$ l of 10  $\times$  PCR reaction buffer (100 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% (w/v) Triton X-100, 0.2 ml BSA, 2.5 U of Taq DNA polymerase (Finnzyme), 200  $\mu$ M of each dNTP, 2 mM forward primer, and 2 mM reverse primer. The complete reaction mixture was incubated in a gradient thermocycler (Eppendorf). The PCR temperature profile used was: 94°C for 3 min, then 30 cycles consisting of 94°C for 1 min, 52°C for 2.0 min, 72°C for 2 min, and finally an extension step at 72°C for 6 min. PCR products were analyzed by electrophoresis on 0.8% (w/v) agarose TAE-gels. PCR products were purified using a PCR purification kit (Genei), then cloned into pGEMT vector (Promega) following the manufacturer’s instructions. The ligated product was transformed into *Escherichia coli* competent cells, and the transformed cells were obtained on ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranose), and IPTG (isopropyl- $\beta$ -D-thiogalactoside) containing LB agar plates. Positive clones were picked by blue/white selection and checked for size of the right insert by PCR.

#### Sequence analysis

The 16S ribosomal RNA gene sequence obtained from the actinobacterial isolates was compared with other representative sequences by using NCBI megaBLAST. Taxonomic affiliation of the sequences was retrieved from *classifier* program of ribosomal database project (RDP-II) (Wang et al. 2007). RDP-II hierarchy was based on the new phylogenetically consistent higher-order bacterial taxonomy proposed by Garrity et al. (2007). Multiple alignments of these sequences were carried out by ClustalW 1.83 version of EBI ([www.ebi.ac.uk/cgi-bin/clustalw/](http://www.ebi.ac.uk/cgi-bin/clustalw/)) with 0.5 transition weight. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). Phylogenetic trees were constructed in MEGA 4 version ([www.megasoftware.net](http://www.megasoftware.net)) using neighbor-joining (NJ), minimum evolution (ME), and unweighted pair group method with arithmetic mean (UPGMA) algorithms (Tamura et al. 2007). Interior NJ and ME trees were constructed by using kimura 2-parameter model. The sequence data of the representative strains of each group were deposited in GenBank under the accession numbers EF417875, EF428028, EF428029, EF428030, EF428031, EF428032, EF428033, EU19923, EU199242, EU199243, EU417875, EU563352, and EU563351.

#### DNA hybridization assay

Culturable bacterial community of habitat water was compared with cultured bacteria from brittle star and host sponge. Based on the analysis, host specific bacteria and ecotypes of sponge-associated cultured bacteria were determined. Specific probe for DNA hybridization assay was developed from 16S rRNA gene sequences of seven most common cultured bacterial groups. Sequences were aligned and probes were designed to hyper-variable regions observed in alignments (Table 1). Oligonucleotide probes specific for each of the isolates were designed by choosing a 20 bp region for which variability was very high with respect to the closet phylogenetic groups and for which there was no match with any other sequence in the data banks. BLAST-search was performed to verify specificity of probe sequences. All probes were checked with PROBE\_MATCH tool of the ARB package and verified

with probe match program of ribosomal database project (RDP-II) (Cole et al. 2007). Specificity of probes was cross-checked experimentally against other six cultured bacterial group and sponge metagenomic DNA, respectively. The oligonucleotide probes EUB338 (Amann et al. 1990) and NON338 (Wallner et al. 1993) were used as positive and negative controls, respectively. DNA hybridization assay was performed as per the procedure described by Goodwin et al. (2005) with suitable modifications. Briefly, probes were T-tailed and immobilized on 96-well microtiter plates (Cinek et al. 2000; Kiesling et al. 2002). To immobilize the probes, 100 µl probe mixture was added to each microplate well and dried at 42°C overnight. Hybridization buffer (0.2 µM filtered Milli-Q water, 5 mM NaCl, 0.5 mM Tris, 0.5 mM EDTA, 10% SDS, 20–40% formamide), high stringency wash buffer (3 M tetramethyl ammonium chloride buffer, 0.1% SDS) and conjugate diluent (5X sodium saline citrate, 10% SDS, 22% formamide) were prepared, and hybridization was performed at 50°C and 60°C using 15 µl of PCR amplicons. Hybridized plates were treated with streptavidin-POD conjugate (Genetix) and colorimetric HRP substrate. A positive yellow color was measured on plate reader at 450 nm. Replicate wells were run by every reaction to validate the results. A reaction was considered positive for sample:blank ratios  $\geq 3$ . These results were also used to analyze total and group-specific colony counts.

## Results and discussion

### Culturable bacteria

In the previous study, we have isolated seven morphotypes from *D. nigra* using selective actinomycetes media

**Table 1** Oligonucleotide probes used in this study

Probes	Target (positions)	Sequences 5' → 3'
EUB338 <sup>a</sup>	Bacterial 16S rRNA (338–335)	GCT GCC TCC CGT AGG AGT
NON338 <sup>b</sup>	Negative control	ACT CCT ACG GGA GGC AGC
ALT734 <sup>c</sup>	MSI026 16S rRNA (734–754)	GTGTGGGTAGCGAACAGGAT
MAR82 <sup>c</sup>	MSI032 16S rRNA (82–102)	AATACCCATGAGGCTTGACG
MIC703 <sup>c</sup>	MSI033 16S rRNA (703–723)	TCCACGCTCTCAAAGGAACT
SAC605 <sup>c</sup>	MSI039 16S rRNA (605–625)	TCGATACTGACGCTGAGGTG
ROS46 <sup>c</sup>	MSI042 16S rRNA (46–66)	GTAAAGCTCCTTCGCCTGTG
ACT792 <sup>c</sup>	MSI051 16S rRNA (792–812)	ACTAGGTGTGGGCGACATTC
PSE171 <sup>c</sup>	MSI057 16S rRNA (171–191)	ACCAACAGAATAAGCACCGG

<sup>a</sup> Bacterial 16S rRNA (338–355) specific for all eubacteria

<sup>b</sup> Negative control probe

<sup>c</sup> Probe name indicates the phylogenetic group closet to each isolate based on 16S rRNA sequences of the respective isolate, and numbers indicate the position of the first base in the 16S rRNA gene (*Escherichia coli* numbering system) for which the probe was designed to target

(Selvin et al. 2004). Based on the pre-determined cultural characteristics of closely related strains of phylogenetic tree and biochemical composition of host sponge, new media compositions were developed. In the preliminary experimentations, the newly developed media compositions were not suitable for the isolation of obligatory sponge endosymbionts due to the contamination of fast growing habitat bacteria. Therefore, the contaminants were eliminated using nystatin/nalidixic acid as selective inhibitor of habitat bacteria. However, subsequent isolation on the media enriched with the selective antibiotic inhibitor did not produce any bacterial colony after 45 days of incubation at 25°C. Therefore, the media was further enriched with selective inducers such as alpha-butyrolactone and host sponge extract. In the present study, we have obtained 145 CFU from host sponge, 96 CFU from brittle star, and 112 CFU from habitat water using novel cultivation approaches. The cultivation potential of sponge-associated bacteria in various media composition is depicted in Fig. 1. Invariably, the number of morphotypes was considerably increased in the MSA enriched with sponge extract, antibiotic, and autoinducers. The order of morphotypes diversity was declined toward MMA<SA<MNA<HA<AA<CSA<RH<FT<MA<EA<TA<PA<ANA. Complete enrichment seems to be an effective composition which increased the cultivation potential considerably. Based on the colony counts, the total culturable bacterial community was extrapolated as  $1.45 \times 10^5$  CFU/cm<sup>2</sup> area in host sponge,  $9.6 \times 10^4$  CFU/g tissue in brittle star, and  $1.12 \times 10^5$  CFU/ml in habitat water (Table 2). Actinobacteria was the major group (46%) of culturable bacterial community associated with *D. nigra*.

**Table 2** Total culturables (TC) and group specific colony counts

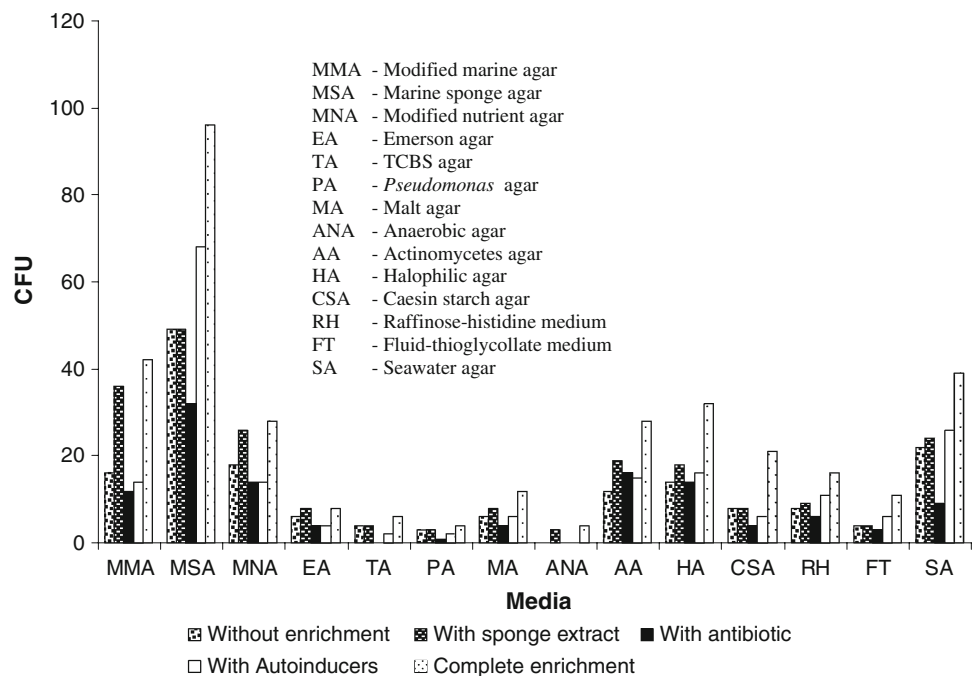
Counts	HS (cfu/cm <sup>2</sup> area)	BS (cfu/g tissue)	HW (cfu/ml)
TC	$1.45 \times 10^5$	$9.6 \times 10^4$	$1.12 \times 10^5$
MSI026	$2.2 \times 10^4$	$3.9 \times 10^3$	–
MSI032	$2.4 \times 10^4$	–	$6.3 \times 10^4$
MSI033	$2.6 \times 10^4$	–	–
MSI039	$1.7 \times 10^4$	$2.8 \times 10^3$	–
MSI042	$2.4 \times 10^4$	$2.9 \times 10^3$	–
MSI051	$1.6 \times 10^4$	–	–
MSI057	$1.4 \times 10^4$	–	$4.8 \times 10^4$

HS Host sponge, BS Brittle star, HW Habitat water

In this group, *Micromonospora* sp. MSI033, *Saccharomonospora* sp. MSI039, and *Streptomyceae* comprised 18%, 12%, and 11% of culturable bacterial community associated with host sponge, respectively. Other bacterial associates were *Marinobacter* (17%), *Roseobacter* (17%), *Alteromonas* (15%), and *Pseudomonas* (10%).

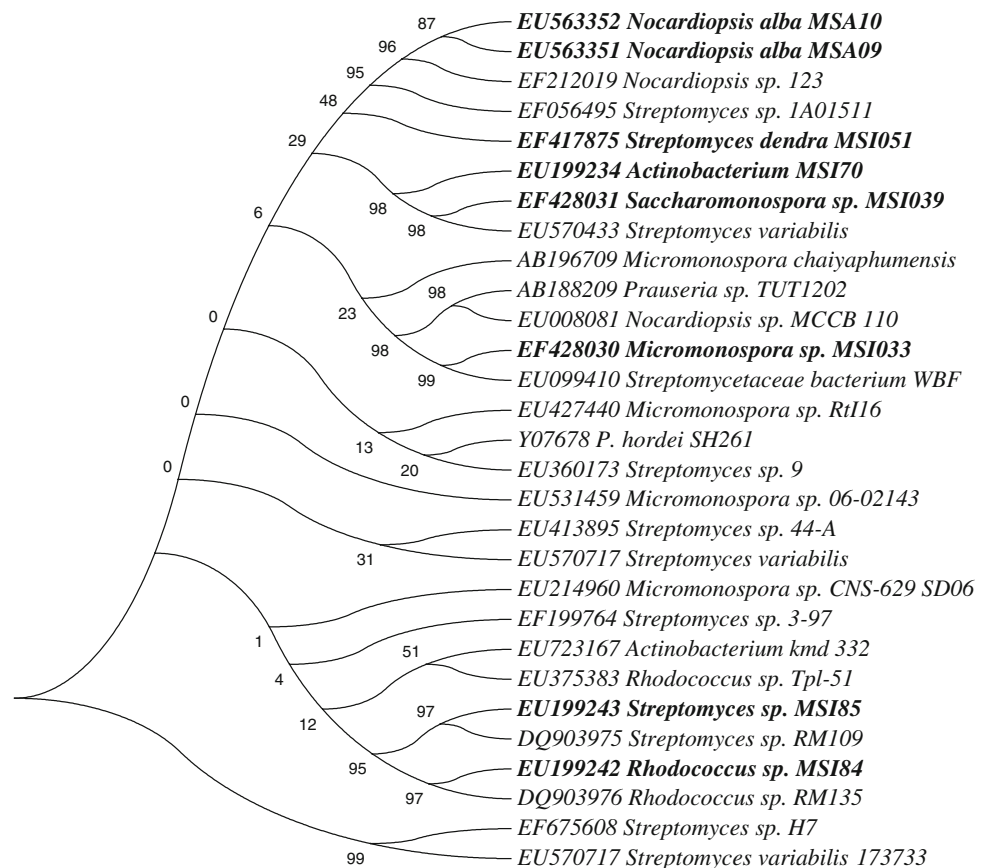
It has been suggested that the growth media should resemble as much as possible the conditions that prevail inside the sponge mesohyl. Recently, it has been realized the production of acyl homoserine lactones (AHLs) by bacteria associated with marine sponges (Michael et al. 2004). Many gram-negative bacteria utilize AHL-mediated signaling systems to communicate with one another (Fuqua et al. 2001). 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

**Fig. 1** Growth pattern sponge (*D. nigra*) associated bacteria on various growth media



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 (Boettcher and Ruby 1995), relatively little is known about the occurrence of AHLs in marine bacterial endosymbionts. However, it has been realized that the addition of AHLs and cyclic AMP (Bruns et al. 2002) or siderophores (Guan et al. 2000) to marine growth media increased bacterial culturability and growth, respectively. The present findings envisaged that the cultivation potential of sponge bacteria could be increased considerably by the addition of selective inducers (AHLs and sponge extract) and regulators (antibiotics). In a previous report, 35% of the supplemented media demonstrated CFU recoveries from marine sponges that were greater than those of the unamended control plates (Olson et al. 2000). New fermentation technologies were developed for symbionts that are not cultivable using the existing media (Piel et al. 2005). Some sponge isolates have been cultured (Osinga et al. 1998), but it remains uncertain whether these are associated bacteria or symbionts. Therefore, in the present study, obligate symbionts were detected using DNA hybridization assay. This is the first report that envisages the culturable obligate symbionts of a marine sponge from Indian coast.

**Fig. 2** UPGMA bootstrapping phylogenetic tree of cultured actinobacterial isolates and their closest NCBI (megaBLAST) relatives based on the 16S rRNA gene sequences. Bootstrap values calculated from 1,000 resamplings using neighbor joining are shown at the respective nodes when the calculated values were 50% or greater



Blast search and phylogenetic analysis based on 16S rRNA gene sequences

Taxonomic affiliation of the 16S rRNA sequences of the isolates was retrieved from *classifier* program of RDPII. The 16S rRNA sequence of eight representative actinobacterial isolates were blasted using megablast tool of GenBank (<http://www.ncbi.nlm.nih.gov/>). Based on the homology and similarity, the isolates were classified as *Micromonospora* sp. MSI033, *Saccharomonospora* sp. MSI039, *Streptomyces* sp. MSI085, a new strain *Streptomyces dendra* MSI051 and an unidentified actinobacterium MSI070, *Nocardioopsis alba* MSA10 and MSA09, and *Rhodococcus* sp. MSI084. Representative of maximum homologous (97–99%) sequences of each isolate was obtained from *seqmatch* program of RDPII and was used for the reconstruction of phylogenetic affiliation (Fig. 2).

#### Detection and grouping of sponge-associated bacteria

Microplate analysis was performed on DNA samples from host sponge, brittle star, and habitat water. The analysis was performed only between the seven representative bacterial isolates of host sponge, brittle star, and habitat water. In addition, cross checking was performed on each probe

against other 352 DNA samples from sponge-bacterial isolates to confirm probe specificity and group specificity (data not shown). All probes were determined as strain specific, and no cross hybridization was observed among the isolates and metagenomic DNA of host sponge. Highest ratio of probe hybridization was observed for the isolate MSI033 (*Micromonospora*) and lowest ratio for MSI057 (*Pseudomonas*) in the host sponge (Table 3). In general, the ratio is an index of cell numbers in the samples. The host sponge retained maximum number of cells compare to brittle star and habitat water. The association of MSI026 (*Alteromonas*), MSI039 (*Saccharomonospora*), and MSI042 (*Roseobacter*) with brittle star indicated that they might be general symbionts. The isolates MSI032 (*Marinobacter*) and MSI057 (*Pseudomonas*) might be sequestered in the sponge tissue from habitat water during filter feeding. Specific symbionts of host sponge were MSI033 (*Micromonospora*) and MSI051 (*Streptomyces*). In this group, *Streptomyces dendra* sp. nov. (MSI051) was determined as a potential producer strain (Selvin 2009). The polyketide synthase gene type II (GenBank accession number EF417875) in the MSI051 ultimately increased the scope for combinatorial biosynthesis and genetic engineering for the production of new antibiotics. Sponge-associated *Actinobacteria* have been reported to be prolific source of secondary metabolite producers (Selvin et al. 2004; Montalvo et al. 2005) and suggested to attribute largely to the chemical defense mechanisms of their host sponges against predators with biologically active compounds (repellents) and biofouling (Meyer and Kuever 2007 and references therein). In the present study, we demonstrated the *Actinobacteria* as the major culturable bacteria community of marine sponge *D. nigra*. The isolate MSI057 (*Pseudomonas*) was found to produce psychrophilic alkaline lipase (Kiran et al. 2008). The functional role of lipase in the host sponge is not known. Based on Gram reaction/biochemical characteristics and DNA hybridization assay, the common isolates of three samples were typed into seven groups. The interaction between the host sponge and members of its symbiont community is likely to have important ecological and evolutionary implications (Piel et al. 2005). Though a diverse assemblages of bacteria present in the habitat, the host sponge perhaps restricts the number and abundance of obligate symbionts (Hill et al. 2006).

Association of culturable actinobacteria including *Micromonospora* and *Streptomyces* was reported from *Haliclona* sp. (Jiang et al. 2007). *Micromonospora* have been isolated previously from a marine sponge *Hymeniacidon perleve* by Zhang et al. (2006). However, the association of *Saccharomonospora* was seldom reported from marine sponges. Maldonado et al. (2005) described the “*Micromonospora–Rhodococcus–Streptomyces*” group seems to be ubiquitous in cultured actinobacteria from

**Table 3** Detection of probe specific bacteria in HS, BS, and HW samples

Probe	Value ratio sample/blank <sup>a</sup>				
	HS	BS	HW	EUB338 <sup>b</sup> Positive control	NON338 <sup>c</sup> Negative control
MSI026	42	12	1	0.9	1
MSI032	38	1	26	1	1
MSI033	50	1	1	1	1
MSI039	32	16	1	1	1
MSI042	36	24	2	0.8	1
MSI051	42	1	1	1	1
MSI057	28	2	18	1	1

HS Host sponge, BS Brittle star, HW Habitat water

<sup>a</sup> Value ratio sample/blank  $\geq 3$  is positive

<sup>b</sup> Bacterial 16S rRNA (338–355) specific for all eubacteria

<sup>c</sup> Negative control probe

marine environments. Based on the present findings, “*Micromonospora–Saccharomonospora–Streptomyces*” group seems to be major culturable actinobacteria in marine sponge *D. nigra*. Domain-specific universal bacterial primers were used to amplify the 16S rDNA gene from genomic DNA that had been extracted from sponges and the surrounding water of Caribbean sponge, *Chondrilla nucula* (Hill et al. 2006). It was also reported that highly diverse bacterial communities including some percentage appear to be specialized for the sponge habitat. Burja et al. (1999) reported a highly diverse assemblage of more than 200 heterotrophic bacteria isolated from the Great Barrier Reef sponge *Candidaspongia flabellate* based on biochemical characterization and this finding was confirmed by the 16S rRNA sequence analysis. In the present study, the DNA hybridization assay was found to be a reliable alternate over other hybridization methods.

The present study was aimed to retrieve sponge-associated and sponge-specific bacteria by novel cultivation approaches. The media rationalized with host sponge extract and alpha-butyrolactone as autoinducer increase the cultivation potential of sponge-associated bacteria. The supplementation of host sponge extract with antibiotics selectively screened against the surrounding seawater (habitat) bacteria increase the scope of retrieving sponge-specific bacteria in culture. Albeit the present study demonstrated the possible retrieval of sponge-associated and sponge-specific bacteria in culture, the number of isolates retrieved might perhaps be a small percent of total microbial symbionts. It has been established that cultivation approaches could retrieve 1% of total microbial symbionts and the remaining unculturable majority require culture independent approaches. The metagenomic approaches to

explore the unculturable microbial majority of *D. nigra* is under investigation in our laboratory. A limitation of the molecular approach to study sponge-associated bacteria is the possibility of contamination by bacteria from the surrounding seawater and/or associated with invertebrates living inside the sponge (Wichels et al. 2006; Thiel et al. 2007). The present findings demonstrated the possibility of targeting the cultured sponge-specific bacteria by DNA hybridization assay. Meyer and Kuever (2007) demonstrated that the microbial consortium of marine sponge *Polymastia cf. corticata* and the ambient bacterioplankton were distinctly different, indicative for the low impact of the surrounding seawater on the sponge-microbe associations. However, in contrast, the present findings evidenced that the sponge-associated bacteria represents transient bacterial populations in the surrounding seawater.

Based on the culturable community structure, the sponge-associated bacteria can be typed as general symbionts, specific symbionts, habitat flora, and antagonists. Based on the local distribution of phylotypes of endosymbionts in the host sponge, the microbial symbionts were classified as “sponge associate,” “specialist,” and “generalist” (Meyer and Kuever 2007). Sponges are filter-feeders, sequester seawater-derived microorganisms that might be enriched by the stable and nutritionally rich microhabitat and become part of the sponge-associated microbiota. The host sponge might provide distinct microenvironments as ecological niches for the different bacterial and archaeal populations. The supplementation of host sponge extract might create such nutritional niche required for the growth of sponge-specific bacteria. The present findings envisaged that majority of culturable bacteria are actinobacteria. In conclusion, the cultivation efficiency of sponge-associated bacteria could be increased considerably using autoinducers and regulators as selective enrichment. The present study is the first report on the cultivation and ecotyping of bacteria associated with *D. nigra*. The DNA hybridization assay appeared as unique assay for the analysis of culturable bacterial community in marine sponges.

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