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Retrieval of euryhaline eubacterial and haloarchaeal bionts from nine different benthic sponges: reflection of the bacteriological health of waters of Mandapam, India

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Bacterial bionts from nine sponges from intertidal reaches of Mandapam (9°17'0" N and 7°7'0"E), namely: Petrosia testudinaria, Cinachyra cavernosa, Haliclona sp., Callyspongia fibrosa, Heteronema erecta, Fasciospongia cavernosa, Callyspongia reticutis var solomonensis unidentified sponges (MAMS), and unidentified sponge (NIO3) were enumerated by the acridine orange and viable count in Tryptone Yeast Extract medium (TYE) with 3-25% NaCl. Forty two pure bionts were characterized to generic level according to Bergey's Systematic Bacteriology and sarted into groups using SYSTAT-v.12.01. Diversity, richness and evenness of genera were determined using PRIMER v.5. Direct bacterial counts for sponges, averaged at $5.2\pm2.20\times10^9$ cells/g. Viable counts averaged at $3.31\pm2.60\times10^8$ cfu/g, $3.83\pm2.86\times10^8$ cfu/g and $2.89\pm2.3\times10^8$ cfu/g on TYE, 3% TYE and NTYE, respectively. Predominant Genera were in the order of *Bacillus*> *Corynebacterium* > *Chromohalobacter*. Bionts of Genera *Loktanella* sp., *Pontibacillus* sp., *Planococcus* sp. , *Enterococcus* sp. are retrieved for the first time from sponges of Mandapam. Retrieval of bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* known for pathogenicity to humans, reflects pollution of Mandapam waters by sewage and *Heralds Caution* for safeguarding the waters to ensure sustainability of biota.

[Keywords: Extremophilic-Eubacteria, Euryhaline, Haloarchaea, Bionts, Sponges, Mandapam-waters]

Introduction

Microorganisms, that get access into the interior of the sponge during the efflux and influx of water establish therein as endobionts or exobionts[1]. Bacteria are reported to contribute up to 40% of the sponge biomass (equal to about 10^8 to 10^9 bacteria/g of sponge tissue).

Mandapam regions in India with shallow waters is intertidal and is rich in marine resource[2]. In recent times, the region is reported as a water body for disposal of sewage, industrial effluents and terrestrial runoff [3,4]. Sponges inhabiting such waters due to their feeding habits are expected to sieve in bacteria singly or along with dissolved, particulate organic and inorganic matter, through their ostia into their system of internal channels. The process thus offers an opportunity for the bacteria to establish as bionts of the host tissue. Bacteriological studies related to unveiling the possible role of the retrieved bionts, state that they are expected to exhibit activities that are antagonistic/ protogonistic/ remediative. Studies on retrieval of bacteria from sponges inhabiting the GoM-India have been carried out using culture media having 3.5% NaCl incapable of supporting growth of halophiles requiring higher concentrations of NaCl [5,6,7], although moderately halophilic/halotolerant heterotrophic bacteria, requiring 15-20% NaCl, have been reported from various non hypersaline marine habitats[8,9].

In the light of the above, in this study, we selected nine different sponges, collected from Mandapam , namely *Petrosia testudinaria, Cinachyra cavernosa, Haliclona sp., Callyspongia fibrosa, Heteronema erecta, Fasciospongia cavernosa, Callyspongia reticutis var solomonensis* and two unidentified sponges to retrieve bacterial bionts, using strategies of manipulation of concentration of NaCl

in the nutrient rich growth medium and their characterization to generic level using keys of Bergey's Determinative Biology and SYSTAT v.12.01, and evaluation of their predominance and commonality with respect to the hosts through PRIMER v.5.

Material and Methods

Sponges were collected from Mandapam (situated at 9°17'0" North and 7°7'0") on East Coast in the Gulf of Mannar (GoM), and deposited under the accession code MAM 1, MAM 2, MAM 4, MAM 5, MAM 6, MAM 10, NIO 1, NIO 2 and NIO 3 in the Biological Chemistry laboratory at the National Institute of Oceanography, Dona Paula, Goa-India.

One gram of sponge tissue was excised from the middle of the whole sponge using a sterile scissor, and washed several times in 3% (w/v) NaCl. The sponge samples were aseptrally homogenized in the same medium using a mortar and pestle. The macerate was filtered to remove any solid debris and used for: Enumeration by Acridine Orange Direct Count (AODC) using epiflyarescent microscopy.

To determine the total bacterial counts, a defined volume of the macerate was fixed with 2.5% (w/v) SEM grade glutaraldehyde to a final concentration of 4% (w/v) and stored at 4°C until use. It was filtered through a 25 mm diameter polycarbonate filter (GE Osmonics, Minneapolis, MN) and stained with 200 µl of Acridine Orange (10 mg/mL) solution for 5 mins as described previously[10]. Filters were air dried and mounted in immersion oil onto a microscopic slide. Bacterial numbers were determined using an epifluorescence microscope (Axioplan microscope; Zeiss, Germany). 20-40 quadrats with a minimum of 600 cells were counted, which gives a precision of ±10%. Bacterial cell numbers were calculated using the Generalization: cells/mL = (SC-BC) × CF × F/V wherein, SC is the mean of sample counts/quadrat; BC is the mean of background counts/quadrat; CF is the effective filter area/quadrat area; F is the volume of preservative/volume sample preserved +1; V is the volume of sample filtered.

Macerate was serially diluted, and 10 µl was plated separately onto i) Tryptone Yeast Extract medium (TYE)[11] consisting of (g/l) $MgS0_4$-20; $CaCl_2$-0.2; Tryptone-5; Yeast Extract-3, pH was adjusted to 7, using 1N NaOH, ii) TYE supplemented with 3% NaCl (3% TYE) and iii) TYE with 25% of NaCl (NTYE)[12]. All the chemicals used were from Himedia-India. Plates were incubated at R.T. until colonies were seen on the plates. The bacterial colonies were enumerated to obtain cfu/g of sponge tissue. Morphology of the colonies growing on TYE medium, 3% TYE and NTYE were recorded. Distinct colonies were purified by repeated streaking on corresponding agar medium, designated and maintained as axenic cultures on agar slopes at R.T. Isolates were designated as GUVFPM; GUVFCCM; GUVFHM; GUVFUM; GUVFCFM; GUVFHEM; GUVFCM; GUVFFM; GUVFSM denoting GU (Goa University),V (Velho), F (Furtado), P (*Petrosia*),C (*Callyspongia*), F (*Fasciospongia*),S (Unidentified Sponge), CC (*Cinachyra cavernosa*), H(*Haliclona*), U(Unidentified Sponge), CF(*Callyspongia fibrosa*), HE (*Heteronema erecta*) and M(Mandapam).Morphological and Biochemical characterisation of bacterial bionts. Morphological and biochemical characteric of the isolates were examined using standard bacteriological procedures and individual isolates were identified upto generic level following schemes given in Bergeys manual of systematic Bocleriology.

Statistical Analysis

For every cultured bacterial isolate, the absence and presence of specific phenotypic character, was assessed and rated as 0 (for negative) or 1 (for positive) respectively. Bacteria cultures were sorted out by measuring the similarity and diversity between two bacteria namely, by dividing the size of the intersection by the size of their union as in Jaccard coefficient. Hierarchical rearrangement of clusters was attained through group-average linking method based on Jaccard distance, which measures dissimilarity between sample sets and is obtained by subtracting the Jaccard coefficient from 1 or by dividing the difference of the sizes of the union and

the intersection of two sets by the size of the union. Computations were carried out using the software SYSTAT v.12.01[14] and dendograms were generated to which reflect the phenotypic relationships between members and expressed as a *phenogram.*

Margalef's species richness **(*d*)**, Pielou's eveness **(*J'*)** and Shannon-Wiener's diversity index **(*H'*)** were analyzed using PRIMER v.5, inorder to arrive at univariate measures of bacterial community in sponges.

Results

The Acridine orange direct counts for each of the nine sponges macerates, ranged from 2.3 - 8.7 $\times$ 10^9 cells/g. The total bacterial counts averaged at 5.2 $\pm$ 2.2 $\times$ 10^9 cells/g. Highest count of 8.7 $\times$ 10^9 cells/g was observed in unidentified sponge MAM5 and the lowest count of 2.30 $\times$ 10^9 cells/g in *C.cavernosa* and *H.erecta*. Bacterial morphotypes resolved were cocci, coccobacilli, curved rods, long rods, short rods (Fig. 1).

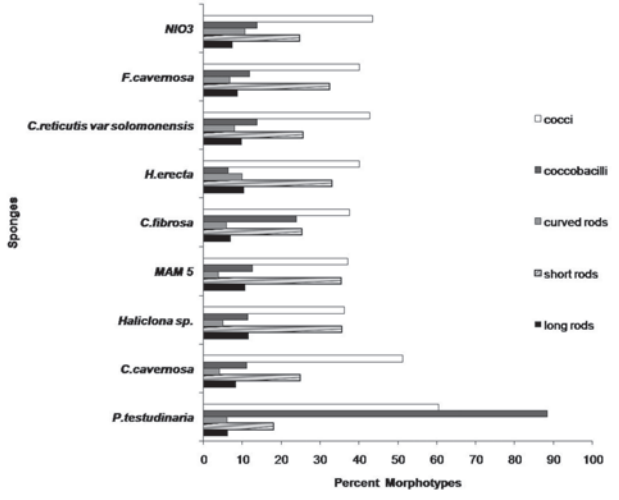

***Fig. 1**–Percent Morphotypes of bacterial bionts revealed by Acridine Orange Direct Count in sponge tissue of Mandapam-India.*

The coccal forms were a maximum of 61% in *P. testudinaria* followed by 51% in *C. cavernosa,* 44% in Unidentified sponge NIO 3, 43% in *C. reticutis var solomonensis,* 40% in *H. erecta* and *F.cavernosa* and a near equal of 38% in *C. fibrosa, Haliclona sp.* and MAM 5. Coccobacillary forms predominated in the sponge *P. testudinaria* at 88% followed by 24% in *C. fibrosa*, 14% in *C. reticutis var solomonensis,* NIO 3 and *F.cavernosa* and a near equal of 12% in sponges *C. cavernosa*, *Haliclona sp.* and MAM 5. Among the rod morphotypes the proportion of the long and curved rods ranged from 6-12% and 3-10% respectively. Short rods on the other hand were the highest with an almost equal in sponges MAM 5 and *Haliclona sp.* at 36% followed by 33% in *H. erecta* and *F. cavernosa,* 25% in sponges *C. fibrosa, C. reticutis var solomonensis,* NIO 3 and *C. cavernosa.*

As seen in Figure 2, total viable counts on TYE ranged from 2$\times$$10^5$ - 8.32 $\times$ 10^8 cfu/g in all the sponges. The counts on TYE averaged at 3.31 $\pm$ 2.60 $\times$ 10^8 at cfu/g. The total viable counts on 3% TYE on the other hand ranged from 2$\times$$10^5$-7.99 $\times$ 10^8 cfu/g with average counts of 3.83 $\pm$ 2.86 $\times$ 10^8 cfu/g in all the sponges. Individual sponges gave a total viable count ranging from 1.2 $\times$ 10^5 - 6.48 $\times$ 10^8 cfu/g on NTYE. The total viable counts on NTYE averaged at 2.89 $\pm$ 2.3 $\times$ 10^8cfu/g. The highest average counts were obtained on 3% TYE.

Greatest diversity of aerobic flora in terms of morphotypes, was observed in TYE media and gradually decreased with the increasing concentration of NaCl. The cream coloured colonies that dominated in NTYE, showed a reddish orange pigment on prolonged incubation. Long incubation periods maximized the viable counts and diversity of the halophiles. Interestingly despite of high colony count,

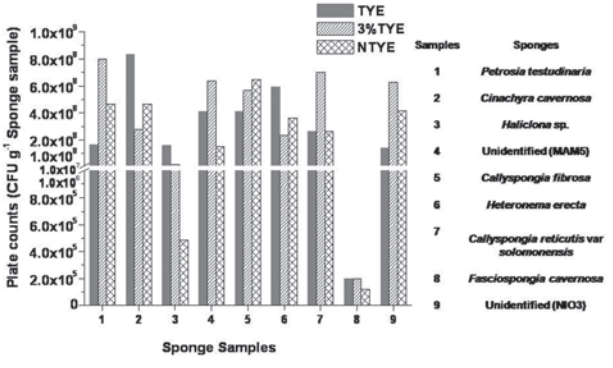

***Fig. 2**–Total bacterial counts in cfu/g of sponge tissue on TYE, 3%TYE and NTYE.*

Table 1– Biochemical potentials and tentative identification of dominant bacterial bionts retrieved and purified from sponges of Mandapam - India

Sponges	Isolates	Retrieval on TYE with NaCl (%)	Pigmentation	Morphotypes/ spores	Gram Character	Motility	Oxidase	Catalase	Glucose	Sucrose	Lactose	Indole	Methyl Red	VP	Citrate	NR	Growth in NaCl (%)			Genera
																	0	3	25	
MAM 1	GUVFPM-1	25	cream	R	-	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	+	*Chromohalobacter* sp. 1
Petrosia	GUVFPM-2	3	cream	SR	+	Nil	+	+	+	Nil	Nil	+	+	Nil	Nil	+	+	+	Nil	*Corynebacterium* sp. 1
testidunaria	GUVFPM-3	3	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	*Bacillus* sp. 1
	GUVFPM-4	3	yellow	RS	+	+	+	+	Nil	Nil	Nil	Nil	Nil	+	Nil	Nil	+	+	+	*Bacillus* sp. 2
	GUVFPM-5	0	white	SR	-	+	Nil	+	+	Nil	Nil	Nil	+	+	Nil	Nil	+	+	Nil	*Enterobacter* sp. 1
	GUVFPM-6	0	beige	SRS	+	+	Nil	+	+	Nil	Nil	+	+	Nil	Nil	Nil	+	+	+	*Pontibacillus* sp. 1
	GUVFPM-7	0	yellow	SR	-	+	Nil	+	+	Nil	Nil	+	+	Nil	Nil	+	+	+	Nil	*Enterobacter* sp. 2
	GUVFPM-8	0	beige	RS	+	+	Nil	+	+	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	+	*Pontibacillus* sp. 2
	GUVFCCM-1	25	CL	SLR	-	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	*Pseudomonas sp.*1
MAM 2	GUVFCCM-2	25	cream	R	-	+	+	+	Nil	+	+	+	+	Nil	Nil	+	+	+	+	*Chromohalobacter sp.* 2
Cinachya	GUVFCCM-3	0	yellow	RS	+	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	+	+	+	+	*Bacillus* sp. 3
cavernosa	GUVFCCM-4	0	cream	R	+	Nil	+	+	+	Nil	Nil	+	+	Nil	Nil	Nil	+	+	+	*Corynebacterium* sp. 2
	GUVFCCM-5	0	orange	RS	+	+	+	+	+	Nil	Nil	+	+	+	Nil	Nil	+	+	Nil	*Bacillus* sp. 4
	GUVFCCM-6	0	yellow	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	Nil	*Bacillus* sp. 5
	GUVFCCM-7	0	white	RS	+	+	Nil	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	*Pontibacillus* sp. 3
	GUVFCCM-8	0	cream	C	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	*Enterococcus* sp. 1
	GUVFCCM-9	0	white	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	Nil	*Bacillus* sp. 6
	GUVFCCM-10	25	cream	R	-	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	+	+	*Chromohalobacter* sp. 3
MAM4	GUVFHM-1	25	cream	RS	+	+	+	+	Nil	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	*Bacillus* sp. 7
Haliclona sp	GUVFHM-2	25	cream	R	-	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	+	+	*Chromohalobacter* sp. 4
MAM5			Pale																	
Unidentified	GUVFUM-1	3	orange	R	+	Nil	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	Nil	*Corynebacterium* sp. 3
sponge	GUVFUM-2	0	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	*Bacillus* sp. 8
MAM6	GUVFCFM-1	0	cream	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	+	Nil	+	+	+	+	*Bacillus* sp. 9
Callyspongia	GUVFCFM-2	0	cream	RS	+	+	+	Nil	Nil	Nil	Nil	+	+	Nil	Nil	Nil	+	+	+	*Bacillius* sp. 10

fibrosa	GUVFCFM-3	0	cream	SR	+	Nil	+	+	+	Nil	Nil	Nil	+	Nil	Nil	+	+	+	+	*Corynebacterium* sp. 4
	GUVFCFM-4	0	cream	RS	+	+	+	+	Nil	Nil	Nil	+	Nil	+	Nil	+	+	+	+	*Bacillus* sp. 11
MAM10	GUVFHEM-1	3	cream	R	+	Nil	+	+	Nil	Nil	Nil	+	Nil	+	Nil	+	+	+	+	*Corynebacterium* sp. 5
Heteronema	GUVFHEM-2	3	cream	SLR	-	Nil	+	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	*Loktanella* sp. 1
erecta	GUVFHEM-3	0	cream	SLRS	+	+	+	+	Nil	+	+	Nil	Nil	Nil	Nil	Nil	+	+	Nil	*Bacillus* sp. 12
	GUVFHEM-4	25	CL	SLR	-	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	*Pseudomonas* sp. 2
	GUVFHEM-5	0	cream	RS	+	+	+	+	+	Nil	Nil	+	+	Nil	Nil	+	+	+	+	*Bacillus* sp. 13
	GUVFHEM-6	0	cream	SR	+	Nil	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	Nil	+	+	+	*Corynebacterium* sp. 6
	GUVFHEM-7	0	cream	C	+	+	+	+	+	Nil	Nil	Nil	+	Nil	Nil	Nil	+	+	+	*Planococcus* sp. 1
NIO1	GUVFCM-1	25	orange	R	-	+	+	+	+	+	+	Nil	Nil	Nil	Nil	+	+	+	+	*Haloarchaea* sp. 1
Callyspongia	GUVFCM-2	0	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	*Bacillus* sp. 14
reticutis var solomonensis	GUVFCM-3	0	CL	SLR	-	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	*Pseudomonas* sp. 3
NIO2	GUVFFM-1	25	orange	R	-	+	+	+	+	+	+	Nil	Nil	Nil	Nil	+	+	+	+	*Haloarchaea* sp. 2
Fasciospongia	GUVFFM-2	25	orange	R	-	+	+	+	+	+	+	Nil	Nil	Nil	Nil	Nil	+	+	+	*Haloarchaea* sp. 3
cavernosa	GUVFFM-3	3	cream	R	-	+	+	+	Nil	Nil	Nil	+	Nil	Nil	+	+	+	+	+	*Chromohalobacter* sp. 5
NIO3	GUVFSM-1	25	orange	R	-	+	+	+	Nil	Nil	Nil	Nil	Nil	Nil	Nil	+	+	+	Nil	*Haloarchaea* sp. 4
Unidentified	GUVFSM-2	0	orange	RS	+	+	+	+	+	+	+	Nil	+	+	Nil	Nil	+	+	+	*Bacillus* sp. 15
sponge	GUVFSM-3	0	CL	SLR	-	Nil	+	+	Nil	+	+	+	Nil	Nil	Nil	+	+	+	+	*Pseudomonas* sp. 4

CL, colourless; R, Rods; RS, Rods with spores; SR, Short rods; SLR, Slender rods; SRS, Short rods with spores; SLRS, Rods fine slender with spores; C, Cocci; VP, Voges Proskauer; NR, Nitrate reduction; (+), present; (Nil),absent; For gram character (+), positive-purple and (-), negative-pink.

the bacterial growth from individual sponges largely comprised of restricted colonial morphotypes, which resulted in isolation and purification of only a maximum of 42 different colonies. Of the total isolates 61.9% were Gram positive, and the remaining 38.09% of the isolates were Gram negative.

Bionts initially retrieved as growth on TYE/ 3%TYE/NTYE, were also able to grow at other concentrations of NaCl (Table 1). and hence categorized as: *marine halophiles* growing at 3% NaCl and *halotolerant or euryhaline* growing at 0-25% NaCl. 78.57% of the bionts associated with all the sponges under study were euryhaline while the remaining 21.42% were marine halophiles.

Morphological and Biochemical Characterization of bacterial bionts and Statistical Analysis

Systat analysis, further sorted the Gram positive and Gram negative bionts based on their biochemical characteristics (Table 1) using distance matrix and UPGMA clustering into 4 and 1 Phena and 1 and 3 Phena**,** respectively as represented in the phenogram (Figure. 3a & b). Thus confirming the identification resolved to generic level, for each retrieved bacterial biont (Table 1) according to keys of Bergey's Systematic Bacteriology[13,15].

Accordingly the 4 and 1 Phena under Gram positive cluster corresponds to 4 Genera under Class Bacilli and 1 Genus under Class Actinobacteria belonging to Phylum Firmicutis (Figure 3a). The retrieved bionts were distributed as in:

Phenon 1 H" Genus *Enterococcus* - GUVFCCM-8 a motile cocci and catalase negative.

Phenon 2 H" Genus *Pontibacillus* - GUVFPM-6, GUVFPM-8 and GUVFCCM-7 are sporulating motile rods, catalase positive and oxidase negative.

Phenon 3 H" Genus *Planococcus* - GUVFHEM-7 a motile cocci, catalase and oxidase positive.

Phenon 4 H" Genus *Bacillus* - GUVFPM-3, GUVFPM-4, GUVFCCM-3, GUVFCCM-5, GUVFCCM-6, GUVFCCM-9, GUVFHM-1, GUVFUM-2, GUVFCFM-1, GUVFCFM-2, GUVFCFM-4, GUVFHEM-3, GUVFHEM-5,

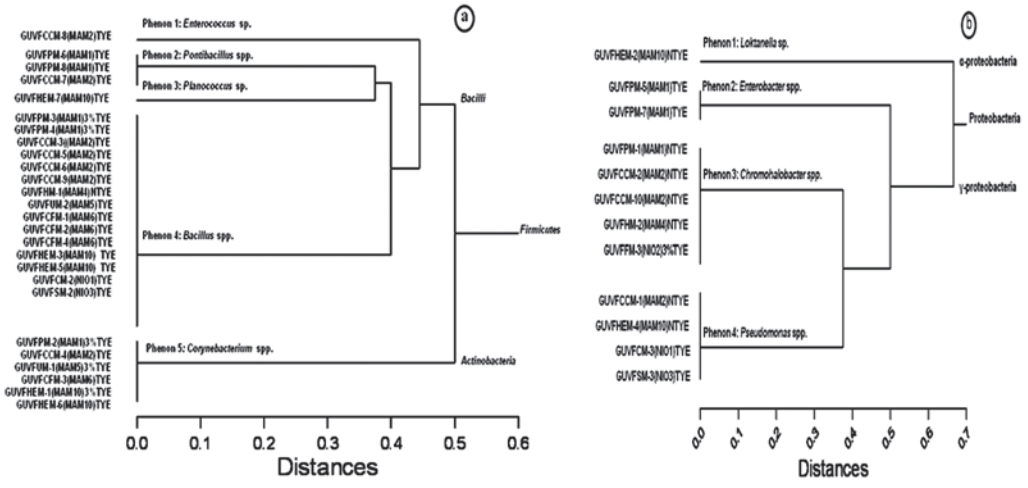

Fig. 3–*Phenogram depicting sorting of morphological and biochemical characteristics of eubacterial bionts retrieved from sponges of Mandapam-India using keys of Bergey's Systematic Bacteriology and SYSTAT v.12.01. (a) Gram positive bacteria H" Cluster A (Phylum: Firmicutes) with Subcluster IA (Class: Bacilli) with 4A Phena (Genera: Enterococcus; Pontibacillus; Planococcus and Bacillus) and Subcluster IIA (Class: Actinobacteria) with 1A Phenon (Genera: Corynebacterium). (b) Gram negative bacteria H" Cluster B (Phylum: Proteobacteria) with Subcluster IB (Class: Alphaproteobacteria) with 1B Phenon (Genera: Loktanella) and Subcluster IIB: (Class: Gammaproteobacteria) with 3B Phena (Genera: Enterobacter; Chromohalobacter and Pseudomonas).*

GUVFCM-2 and GUVFSM-2 are sporing rods, oxidase and catalase positive.

Phenon 5 H" Genus *Corynebacterium* - GUVFPM-2, GUVFCCM-4, GUVFUM-1, GUVFCFM-3, GUVFHEM-1 and GUVFHEM-6, are non motile rods, catalase and oxidase positive.

Similarly the 1 and 3 Phena obtained under Gram negative cluster corresponds to the 3 Genera under Class Alphaproteobacteria and 1 Genus under Class Gammaproteobacteria belonging to Phylum Proteobacteria (Figure 3b). The retrieved bionts distributed as in:

Phenon 1 H" Genus *Loktanella* - GUVFHEM-2 a non motile rod, oxidase and catalase positive, methyl red negative and did not reduce nitrate to nitrite.

Phenon 2 H" Genus *Enterobacter* - GUVFPM-5 and GUVFPM-7 are motile rods, lactose non fermentors, oxidase negative and catalase positive.

Phenon 3 H" Genus *Chromohalobacter* - GUVFPM-1, GUVFCCM-2, GUVFCCM-10, GUVFHM-2 and GUVFFM-3 are motile rods, oxidase Pasitive catalase positive and indole positive.

Phenon 4 H" Genus *Pseudomonas* - GUVFCCM-1, GUVFHEM-4, GUVFCM-3 and GUVFSM-3 are non motile rods, oxidase and catalase positive and did not produce acid from glucose

These bionts belonging to nine Genera showed varied distribution in sponges. The sponge *P. testudinaria,* showed the presence of 62.5% of euryhaline and 37.5% of marine halophilic bionts. As detailed in Table 1, the euryhaline bacteria retrieved from *P. testudinaria* were identified as *Chromohalobacter* spp., *Bacillus* spp. and *Pontibacillus* spp. while the marine halophiles were identified as *Corynebacterium* spp. and *Enterobacter* spp. 70% of isolates associated with sponge *C.cavernosa* were euryhaline bacteria, identified as *Pseudomonas* spp., *Chromohalobacter* spp., *Bacillus* spp., *Corynebacterium* spp., *Pontibacillus* spp. and *Enterococcus* sp. while the remaining 30% were marine halophiles belonging to the Genus *Bacillus* spp. All the isolates associated with sponge *Haliclona* sp. were euryhaline bacteria identified as *Bacillus* spp. and *Chromohalobacter* spp. One of the isolates retrieved from the unidentified sponge MAM 5 was an euryhaline bacterium identified as *Bacillus* spp. while the other, was a marine halophile identified as *Corynebacterium* spp.

All the isolates retrieved from sponge *C. fibrosa* were euryhaline bacteria belonging to the Genus *Bacillus* spp. and *Corynebacterium* spp. 85.7% of the isolates associated with sponge *H. erecta* were euryhaline bacteria identified as *Corynebacterium* spp., *Loktanella* sp. , *Pseudomonas* spp., *Bacillus* spp. and *Planococcus* sp., while the only marine halophile belonged to the Genus *Bacillus*.

Isolates GUVFCM-2 and GUVFCM-3 from *C. reticutis var solomonensis* and isolates GUVFSM-2 and GUVFSM-3 from Unidentified sponge NIO 3 were euryhaline bacteria belonging to the Genus *Bacillus* and *Pseudomonas*. Isolate GUVFFM-3 from sponge *F.cavernosa* belonged to the Genus *Chromohalobacter* and was an euryhaline bacterium.

Isolate GUVFCM from the sponge *C. reticutis var solomonensis*, GUVFFM-1 and GUVFFM-2 from the sponge *F.cavernosa* and the single isolate GUVFSM from the unidentified sponge NIO 3, were tentatively identified as Haloarchaea and they were growing in medium with 25% NaCL, produced the

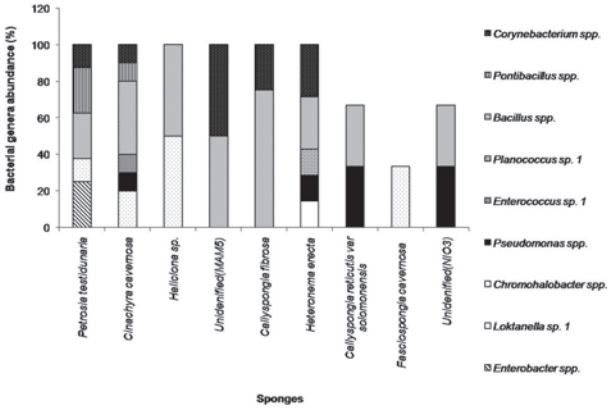

Fig. 4 *–Dominant Genera of culturable bacterial bionts from different sponges.*

orange pigments and showed presence of Glycerol diether moieties, which are chemotaxonomic markers for ascribing the isolates as members of the third domain.

As recorded in Figure 4, bionts of genus *Bacillus*, followed by those of genera *Corynebacterium* predominated 88.9% and 55.6% of sponge samples respectively. *F.cavernosa* is the only sponge having *Chromohalobacter* as its lone Genera.

Statistical measures of diversity

PRIMER v.5 showed highest Genera richness (*d*) and diversity (*H'*) in sponge *C.cavernosa* followed by *Heteronema erecta* and *Petrosia testudinaria* (Figure 5a & c).

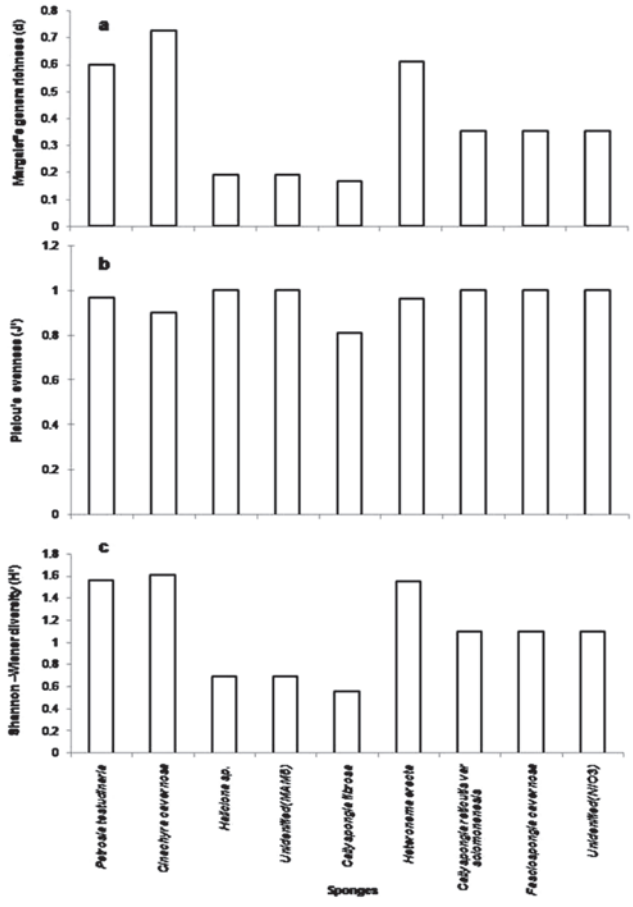

Fig. 5–*(a) Genera richness, (b) Pielou's evenness and (c) Shannon-Wiener diversity of the bacterial community in the different sponges.*

Interestingly the bacterial bionts were evenly distributed in sponges namely *Haliclona* sp., Unidentified sponge (MAM 5), *Callyspongia reticutis var solomonensis, Fasciospongia cavernosa* and Unidentified sponge (NIO 3) as seen in Figure 5b.

Discussion

The total bacterial counts determined using AODC method, averaged at, $5.20 \pm 2.2 \times 10^9$ cells/g and are comparable with counts $2.8 \pm 0.4 \times 10^9$ cells/g reported from other marine sponges[16]. This result clearly supports the finding that sponges provide an econiche wherein several bacterial types dock for safety against predators or harsh environmental conditions and for nutrition[17-20].

Even though our sponges were washed repeatedly with 3% NaCl to get rid of the surface associated bacteria the total viable counts of intimate associates of sponges are 10^3 times higher in comparison to the earlier reports from sponges, of this embayment[7]. This is also substantiated by the total bacterial counts in the same sponge samples. However this may not be unusual as bacterial counts in marine inverterbrates including sponges are reported to be 2-3 folds higher in comparison to the free living/planktonic bacteria in the ambient waters and is a pointer to the significant role played by these micro-organisms in sponges[21].

The isolates retrieved from the different sponge samples under study differed in the requirement of NaCl concentration used for their recovery and hence were either euryhaline or marine halophiles or haloarchea. It was interesting to note that even though culturable bacteria were obtained on all the three isolation media (TYE, 3% TYE and NTYE) from all the sponge samples under study, the euryhaline bacteria predominated the culturable microbial assemblage. Euryhaline bacteria are capable of growth over a wide range of NaCl concentration (between 0-25% NaCl), and are also referred to as 'halotolerant extremophiles'[22].

Sponges through their filter feeding mode of nutrition[23,24] accumulate large amounts of dissolved

organic matter (DOM) from the habitat waters, in their choanocytes. In recent years, assimilation of DOM is ascribed to the sponge–microbe associates that participate in the sponge nutrition by secreting extracellular enzymes that act on the organic matter accumulated within the sponge body, and thereby provide assimilable carbon to the sponge host[25-27]. The relenting osmolal stress owing to the increased concentration of organic compounds in the sponge interiors due to the sequestering of DOM creates a selection pressure on euryhaline bacteria. Given to understand, the tolerance of euryhaline halophiles to high or fluctuating salinities[28], it is reasonable to consider that we found euryhaline bacteria to predominate the cultural assemblage in intertidal sponge species which face abrupt and short term salinity fluctuations.

Physiological flexibility of these isolates[22] and their ability to resist the changing osmolar conditions adds to the selection pressure. Retrieval of euryhaline archaea from these sponges in our study corroborates with the findings of Martins *et al*.[29] who demonstrated the production of compatible solutes to cope osmotic stress which is possibly responsible for establishment of bionts of genera not known to produce osmolytes.

In the present study, we demonstrated *Bacillus* spp. and *Corynebacterium* spp., as the major culturable bacterial community in all the sponges under study. The greater predominance of Gram-positive bacteria is probably attributable to their ability to produce spores under adverse conditions; a physiological adaptation enabling eubacterial bionts to survive within the sponge interiors.

Studies by Devi *et al*.[30] ; Feby and Nair[27]; Vasanthbharathi and Jayalakshmi[6] and other workers have demonstrated the presence of bacteria in Indian sponges with a Single report from sponges of Mandapam by Saravanakumar *et al*.[7] are recard far the first time occurrence and retrieval of euryhaline, halotolerant extremophilic eubacteria (0-20%) and haloarchaeal bionts from nine different benthic sponges of Mandapam in India. Earlier studies on Indian sponges have reported bacteria growing in 0.5-3% NaCl [5-7] whereas in the present study bacterial bionts grew at 0-25% NaCl.

To our knowledge eubacteria from Genera *Loktanella* sp., *Pontibacillus* sp., *Planococcus* sp., *Enterococcus* sp. retrieved by us have not been reported from Mandapam waters possibly, as they could not withstand tidal fluctuations. Retrieval of sponge bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* known for their pathogenicity to humans and others, reflects their occurrence in the habitat waters, possibly accounting to reports describing Mandapam in the GoM, as a sink, receiving high load of organic matter through the indiscriminate discharge of industrial effluents, household wastes, leacheate of solid waste dumps and garbage, animal and human excreta etc. [3,4,31]

The retrieval of bacteria from nine Genera indicates that the sponge tissue provides a suitable substratum for settlement of bacteria in turn reflecting their occurrence in habitat waters.

Conclusion

The study clearly reveals (1) The presence of cohabitating marine halophiles (3% NaCl), halotolerant or euryhaline halophiles (0-25% NaCl) and haloarchaea. (2) The predominance of euryhaline bacteria, capable of coping with osmolal stress in the sponge interiors, possibly due to the sequestration of DOM from the ambient waters of the sponge habitat, which inturn supports co-existence of bionts of Genera unknown to produce osmolytes. (3) New Genera *Loktanella* sp., *Pontibacillus* sp., *Planococcus* sp., *Enterococcus* sp. were retrieved by us, not yet reported from Mandapam waters. (4) That retrieval of sponge bionts of Genera *Enterococcus*, *Corynebacterium*, *Enterobacter* and *Pseudomonas* reflects the entry of bacteria, known for pathogenicity to humans, into waters of Mandapam, through sewage and other pollutants. (5) Microbial pollution of Mandapam waters and *Heralds Caution* for taking measures for safeguarding the waters and ensuring sustainability of biota.

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