Two marine sponges-associated cultivable bacteria: Diversity and biological activities

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ABSTRACT. Marine sponges harbor diverse bacterial communities. Sponge associated bacteria produce potential secondary metabolites of medical use. Little is known about sponge associated diversity from red sea therefore, we have collected two sponge samples i.e., *Pione vastifica* and *Siphonochalinna siphonella* collected from north of red sea in Obhur region, Jeddah Saudi Arabia. By using culture dependent method, we have isolated 95 different bacterial species from two marine sponge samples. These marine bacteria were screened for their antagonistic potential against fungal pathogens (*Phytophthora capsici* and *Pythium ultimum*) and human pathogenic bacteria (*E. coli*, Methicillin-resistant *Staphylococcus aureus*, *E. faecalis*, and *P. aeroginosa*). Among all 37 (39%) marine bacteria showed inhibition against oomycetes, only 27 (28.4%) exhibited antibacterial activity while 19 (20%) exhibited both antifungal and antibacterial activities. These bacterial strains were further screened for enzyme production (cellulase, protease, lipase, and amylase). Most of the strains were positive for production of lipase enzyme. These antimicrobial activities and enzyme production suggest their role in marine sponge as protecting against different marine pathogens. Taxonomic and phylogenetic analyses on the basis of 16S rRNA gene sequences showed that dominant phylum was γ-Proteobacteria. Our results highlighted that marine sponges are potential source of marine bacteria producing antimicrobial

metabolites and enzymes of pharmaceutical and industrial significance.

Keywords: Marine sponges; 16S rRNA gene sequence; Anti-microbial activity; Enzymatic potential

INTRODUCTION

Due to an increase in Multi Drug Resistant (MDR) bacteria, health risk possesses to human population. Therefore, there is need for effective antimicrobial compounds, especially antibiotics effective against these resistance bacteria. Marine environment is currently a promising source of potent bioactive chemicals; especially invertebrates are known to harbor diverse microbial communities due to their filter-feeding habit and symbiosis (Zhang et al., 2015). Mutualistic relationship between marine sponges and their associated microbes is important for both of them. As sponge provide place for colonization, shelter from predator and nutrients to microbe and in turn by products of sponge are eliminated by these microbes as well as bioactive compounds excreted by these microbes help against different microbial disease (Taylor et al., 2007).

Microbial communities of marine invertebrates reported to provide biologically active compounds of biotechnological and pharmaceutical significance (Blunt et al., 2016). Marine sponges are hosts for symbiotic microorganisms and recently many bioactive compounds were isolated from these microorganisms. It has been reported that more than 15,000 natural compounds with more than 8000 new compounds had been isolated from marine invertebrate where 30% discovered from marine sponges (Koopmans et al., 2009; Brinkmann et al., 2017). Natural products derived from marine sponges are diverse in function ranging from anti-inflammatory, antiviral, antitumor, immunosuppressive to antibiotic (Imhoff et al., 2011). Most of these bioactive compounds isolated from marine sponges are in fact product of associated microbes (Khan et al., 2014).

To get knowledge of sponge and associated bacterial community's interactions, it is important to identify, study diversity and characterize marine sponges' associated microbial communities. Both culture dependent and culture-independent techniques reported diversity of bacterial phyla associated with marine sponges (Taylor et al., 2007; Öztürk et al., 2013) and until now 26 different phyla have been reported from marine sponges (Hentschel et al., 2002; Lee et al., 2011; Webster et al., 2001). Studies have been also performed to isolate sponge associated bacteria and screened them for the production of active metabolites (Anand et al. 2006; O'Halloran et al. 2011).

However, there is lack of investigations for bacterial association with marine sponges from region of Saudi Arabia. Therefore, the aim of present study is isolation and identification of bacteria from two selected species of marine sponges namely *Pione vastifica* and *Siphonochalina siphonella* and furthers their screening for antibacterial and antifungal activities as well as characterizes them for their enzymatic potential.

MATERIALS AND METHODS

Sample collection

Two sponge samples were collected by SCUBA at the depth of 20-30m from the Jeddah, Red Sea. These sponge samples were identified as *Pione vastifica* and *Siphonochalina siphonella*, by Dr. Mohsin from marine science department King Abdul-Aziz University. After collection these sponge samples were covered by seawater inside sterile ziploc plastic bag and transported immediately to the laboratory for bacterial isolation.

Isolation of bacteria from sponge samples

The sponge samples were cut into small pieces and approximately 1g of each sponge sample was finely cut into small pieces and ground with a sterile mortar and pestle. Aliquots (0.1ml) were 10-fold diluted (10^{-3}, 10^{-4} and 10^{-5}) in sterile filtered seawater (FS) and 0.1 ml aliquots will be spread on to isolation media. Isolation media were half strength R2A (½ R2A), half Tryptic soy agar (½ TSA), half nutrient agar (½ NA), in sea water and marine agar (MA) using distilled water. Cyclohexamide (50µg/ml) was added to inhibit the growth of fungi. Plates were incubated at 25°C for 5-7 days. Bacterial colonies were purified by transferring onto new plates.

Using 1/10 R2A medium in FS isolated strains were sub-cultured and stored at -70°C in ½ R2A broth in FS containing 15% (v/v) glycerol for further use.

DNA extraction and 16S rDNA gene analysis

For identification of isolated bacterial strains, genomic DNA was extracted, and strains were subjected to 16S rDNA gene analysis. For extraction of genomic DNA, genomic DNA extraction kit (Qiagen) was used. The 16S rDNA gene fragment of almost 1.5 kb was amplified from the extracted bacterial DNA using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT -3') and amplifications were performed under PCR conditions described previously (Bibi et al., 2012). PCR products were purified using PCR purification kit (Qiagen) according to the manufacturer's instructions and sequenced commercially (Macrogen, South Korea). Bacteria were identified after blast searches of their 16S rRNA gene sequences obtained using the EzTaxon server (https://www.ezbiocloud.net) (Kim et al., 2012). Phylogenetic status of the isolated bacteria was confirmed using CLUSTALX (Thompson et al., 1997) and BioEdit software (Hall, 1999) was used to edit the sequences. In MEGA6 Programme, neighbor-joining method with bootstrap values based on 1,000 replications was used for construction of the phylogenetic tree based on the 16S rRNA gene sequences (Tamura et al., 2013).

Analysis of antagonistic activity against oomycetes

Antifungal activity of isolated bacteria was evaluated by checking inhibition of the growth of fungal pathogens using a confrontation bioassay as described previously (Bibi *et al.*, 2012). The plant pathogenic oomycetes *Phytophthora capsici* and *Pythium ultimum* were obtained in this laboratory and used in bioassay. Bacteria were screened for their antagonistic activity on modified PDA media using cross streak method (Bibi *et al.*, 2012). All bacterial isolates were streaked on modified PDA media containing ½ PDA medium with ½ R2A in sea water. Mycelial disc of 6 mm of freshly cultured fungal pathogen was placed in the center of plate and bacteria were streaked perpendicular to edges of plate at 4 cm distance and incubated for 3–5 days at 28°C. Bacteria positive for antagonistic activity were checked twice and activity was evaluated by measuring the zone of inhibition around each bacterial streak.

Screening of bacteria for antibacterial activity

Bacteria isolated from marine sponges were screened for antibacterial activity using deferred antagonistic assay. Bacterial isolates from this study were grown at 28°C for 24 hrs on ½ R2A media and then overlaid with 0.1% soft agar mixed with test strains. All test strains were diluted to final concentration A600 =0.1. Plates were again incubated at 28°C for 48 hrs and the zone of inhibition was documented. The test strains of bacteria (*Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270 and *Pseudomonas aeruginosa* ATCC 27853) were pregrown in LB broth at 37°C.

Evaluation of hydrolytic enzymatic activity

Bacterial isolates from two sponges were further evaluated for their hydrolytic enzyme production. To check protease activity, skim milk ½ R2A agar plates were used. Positive isolates were exhibiting protease production and made clear zone on plates. Starch media was used to check amylase production of bacterial strains. Hydrolysis of starch was seen on agar plates as a clear zone by positive strains (Kumar et al., 2012). To check lipolytic activity of strains, ½ R2A agar media supplemented with tributyrin was used. After incubation at 28°C for 48 hrs positive isolates showed clear zone around as tributyrin hydrolyszed. For cellulase activity, bacteria were streaked on CMC agar (carboxy methyl cellulose agar) media and incubated at 28°C for 48 hrs. Plates were then flooded with congo red (0.1%) solution and placed on orbital shaker for 45 min and then rinsed with 1M NaCl (Hendricks et al., 1995). Isolates active for cellulose production were observed as making clear zone on CMC agar plates.

Nucleotide sequence accession numbers

Nucleotide sequences of the bacteria isolated from sponges were deposited in the GenBank database under accession numbers KY436424–KY436445.

RESULTS

Isolation of rhizo and endophytic bacteria from halophytes

Two sponge samples, *P. vastifica* and *S. siphonella* were collected and included in this study for isolation and identification of bacteria associated with them. **(**Figures 1a and 1b).

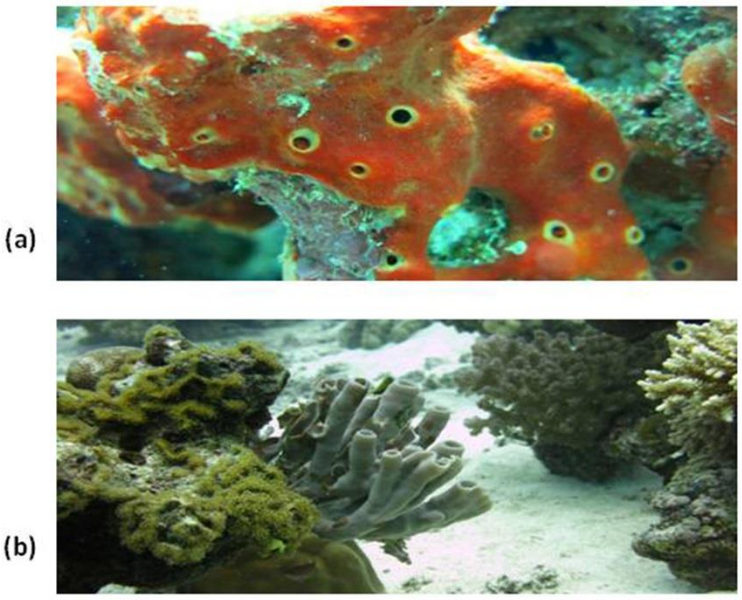

Figure 1. Samples of two marine sponges, (a) *Pione (Cliona) cf. vastifica* and (b) *Siphonochalina siphonella* collected from Red sea.

Four different types of media i.e., ½ R2A, ½ TSA, ½ NA, MA were used for culturing of bacteria. Number and type of bacteria differed on different media by counting colony forming units (CFU) and looking at morphology of bacterial colony (data not shown). High numbers of bacteria were seen on ½ R2A followed by ½ TSA while low numbers of bacteria were seen on both ½ NA and MA indicating that ½ R2A and ½ TSA are favorable for isolation of bacteria from sponge samples. Low number of bacteria on these media may be due to high concentration of nutrients in MA and ½ NA didn't favor growth of marine bacteria from marine sponges. For both sponge samples 20-40 times more CFU were isolated on ½ R2A as compare to other three media. Therefore, concentration and choice of culturing media for isolation of bacteria is very important to provide them with conditions optimal for their growth. A total of 95 bacteria were isolated from these two marine sponges using four different culturing media (Table 1).

Table 1. Distribution of total number of bacteria and antagonistic one from two marine sponge samples.

Sponges	Isolates	Antagonists	Antagonists (%)	Dominant phylum
Pione vastifica	57	24	42.1	*γ Proteobacteria*
Siphonochalinna Siphonella	38	13	34.2	*γ Proteobacteria*
	95	37	52	

Phylogenetic analysis of antagonistic bacteria based on 16S rRNA gene sequence

All these bacteria were identified by using 16S rRNA gene sequence analysis. From sponge *P. vastifica*, 9 different genera i.e., *Vibrio*, *Halomonas*, *Alteromonas*, *Staphylococcus*, *Pseudoalteromonas*, *Tenachibaculum*,

Spongiobacter, *Bacillus*, *Pseudovibrio* were identified and further belong to 3 different classes (*γ-Proteobacteria*, *Firmicutes* and *Flavobacteria*) where dominant class was *Actinobacteria* (42%) (Figure 2a).

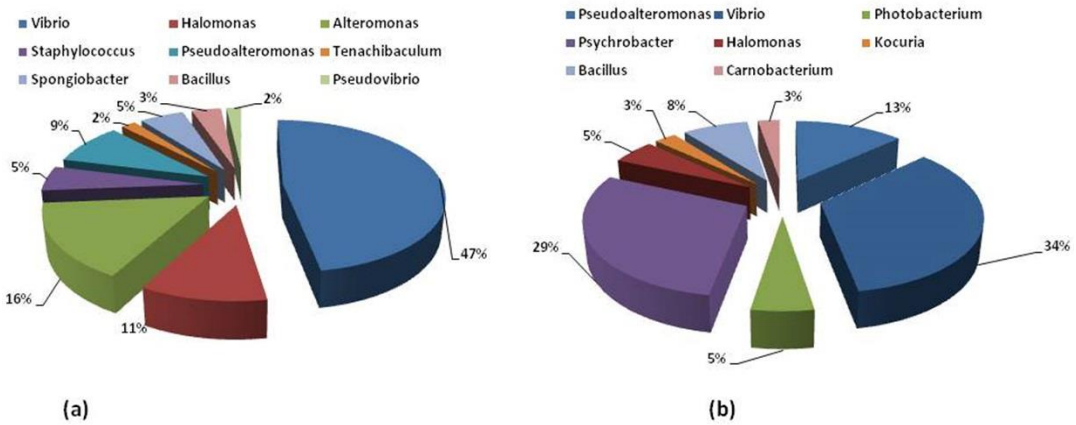

Figure 2. Percentage composition of different genera of isolated bacteria from two marine sponges on the basis of 16S rRNA gene sequence similarity. (a) *Pione (Cliona) cf. vastifica* and (b) *Siphonochalina siphonella*.

From *S. siphonella*, eight different genera of bacteria were identified namely, *Pseudoalteromonas*, *Vibrio*, *Photobacterium*, *Psychrobacter*, *Halomonas*, *Kocuria*, *Bacillus*, *Carnobacterium* and belonging to three different classes (*γ-Proteobacteria*, *Actinobacteria* and *Firmicutes*) and dominant class was *γ-Proteobacteria* (34%) (Figure 2b). Sequence similarities of isolated bacteria from 94.2%–100% and 95.7%–100% from *P. vastifica* and *S. siphonella* (Table 2).

Table 2. Taxonomic identification, antifungal and antibacterial activity of bacteria from sponges, *P. vastifica* and *S. Siphonella*

				Antifungal activity[c]		Antibacterial acitivity[d]			
Lab no	**Accession Number**	**Similarity with closest type strain[a]**	**% identity[b]**	***P. capsici***	***P. ultimum***	***P.aerugino sa***	***S. aureus***	***E.co li***	***E.faecal is***
			P. vastifica						
EA250	KY655351	*Vibrio hepatarius* LMG 20362(T)	98.8	+	+	-	-	-	-
EA251	KY655352	*Halomonas denitificans* M29(T)	98.1	+	+	-	+	w	
EA252	KY655353	*Alteromonas marina* SW-47(T)	98.7	-	-	-	-	-	-
EA253	KY655354	*Staphylococcus argenteus* MSHR1132(T)	99.8	++	++	+	++	+	-
EA254	KY655355	*Pseudoalteromonas shioyasakiensis* SE3(T)	99.7	+	+	-	-	-	-
EA255	KY655356	*Halomonas denitificans* M29(T)	98.2	+	++	-	+	+	-
EA256	KY655357	*Vibrio fortis* LMG 21557 (T)	99.7	++	++	-	+	+	
EA257	KY655358	*Vibrio harveyi* ATCC 14126(T)	99.2	-	-	-	-	-	-
EA258	KY655359	*Vibrio shilonii* AK1(T)	99.7	+	+	-	-	-	-
EA259	KY655360	*Vibrio parahaemolyticus*NBRC 12711(T)	99.5	+	+	-	-	-	++
EA260	KY655361	*Vibrio antiquarius* Ex25(T)	99.5	++	+++	-	w	-	++
EA261	KY655362	*Tenachibaculum litopenaei* B-1(T)	99.5	++	++	-	-	-	-
EA262	KY655363	*Pseudoalteromonas shioyasakiensis* SE3(T)	94.3	+	+	-	-	-	-
EA263	KY655364	*Halomonas ventosae* Al12(T)	96.9	-	-	-	-	-	-

EA264	KY655365	*Pseudovibrio denitificans* DN34(T)	100	+	+	-	-	-	+
EA265	KY655366	*Halomonas ventosae* Al12(T)	98.3	++	++	-	+	-	-
EA266	KY655367	*Alteromonas marina* SW-47(T)	99.7	-	-	+	-	+	-
EA267	KY655368	*Halomonas aquamarina* DMS30161(T)	99.1	-	-	+	-	-	-
EA268	KY655369	*Alteromonas marina* SW-47(T)	94.2	+	+	-	-	-	-
EA269	KY655370	*Vibrio Caribbeanicus* ATCC BAA-2122(T)	98.6	-	+++	-	-	-	-
EA270	KY655371	*Vibrio hepatarius* LMG 20362(T)	98.7	-	-	-	-	-	-
EA271	KY655372	*Spongiobacter nickelotolerans* OOP-Ni033-1-1-2(T)	99.5	-	-	-	-	-	-
EA272	KY655373	*Pseudoalteromonas shioyasakiensis* SE3(T)	95.7	-	-	-	-	-	-
EA273	KY655374	*Spongiobacter nickelotolerans* OOP-Ni033-1-1-2(T)	99.5	-	-	-	-	-	-
EA274	KY655375	*Alteromonas marina* SW-47(T)	93.9	+	+	-	-	-	-
EA275	KY655376	*Alteromonas macleodii* ATCC 27126(T)	99.8	+	+	-	-	-	-
EA276	KY655377	*Spongiobacter nickelotolerans* OOP-Ni033-1-1-2(T)	98.8	++	++	-	-	-	-
EA277	KY655378	*Alteromonas marina* SW-47(T)	99.1	+	+	-	-	+	-
EA278	KY655379	*Bacillus pumilus* ATCC 7061(T)	99.8	-	-	-	w	++	-
EA279	KY655380	*Vibrio antiquarius* Ex25(T)	99.7	-	-	-	-	++	-
EA280	KY655381	*Vibrio alginolyticus* NBRC 15630(T)	99.5	-	-	-	-	-	-
EA281	KY655382	*Vibrio Owensii*LMG 25443(T)	99.8	-	-	-	-	-	-
EA282	KY655383	*Vibrio maritimus* R-40493(T)	99.7	-	-	w	-	++	-
EA283	KY655384	*Vibrio neocaledonicus* NC470(T)	100	-	-	-	-	-	-
EA284	KY655385	*Vibrio hepatarius* LNG 20362(T)	98.7	+	+	-	-	++	-
EA285	KY655386	*Vibrio Caribbeanicus* ATCC BAA-2122(T)	96.8	-	-	-	-	-	-
EA286	KY655387	*Vibrio madracius* R-40493(T)	95.6	-	-	-	-	-	-
EA287	KY655388	*Vibrio fortis* LMG 21557 (T)	100	-	-	-	-	-	-
EA288	KY655389	*Vibrio azurius* NBRC 15630(T)	99.7	-	-	-	-	-	-
EA289	KY655390	*Alteromonas gracilis* 9a2(T)	94.9	-	-	-	-	-	-
EA290	KY655391	*Vibrio madracius* R-40493(T)	99.4	-	-	-	-	-	-
EA291	KY655392	*Staphylococcus argenteus* MSHR1132(T)	100	-	-	-	-	-	-
EA292	KY655393	*Vibrio shilonii* AK1(T)	99.7	-	-	-	-	-	-
EA293	KY655394	*Alteromonas macleodii* ATCC 27126(T)	99.4	-	-	-	-	-	-
EA294	KY655395	*Staphylococcus argenteus* MSHR1132(T)	99.7	-	-	-	-	-	-
EA295	KY655396	*Vibrio hepatarius* LNG 20362(T)	98.8	-	-	-	-	-	-
EA296	KY655397	*Alteromonas macleodii* ATCC 27126(T)	99.7	-	-	-	-	-	-
EA297	KY655398	*Vibrio caribbeanicus* ATCC BAA-2122(T)	98.6	-	-	-	-	-	-
EA298	KY655399	*Halomonas meridiana* DSM5425(T)	99	-	-	-	-	-	-
EA299	KY655400	*Pseudoalteromonas shioyasakiensis* SE3(T)	99.4	+	+	-	-	-	-
EA300	KY655401	*Vibrio mediterranei* CIP 103203(T)	99.4	-	-	-	-	-	-
EA301	KY655402	*Bacillus licheniformis* ATCC 14580(T)	99.4	++	+++	-	+	-	-
EA302	KY655403	*Vibrio alginolyticus* NBRC 15630(T)	99.7	-	-	-	-	-	-
EA303	KY655404	*Vibrio neocaledonicus* NC470(T)	99.7	+	+	-	-	-	-
EA304	KY655405	*Pseudoalteromonas shioyasakiensis* SE3(T)	98.4	-	-	-	-	-	-
EA305	KY655406	*Vibrio Caribbeanicus* ATCC BAA-2122(T)	98.7	+	+	-	-	-	-
EA306	KY655407	*Vibrio nereis* ATCC 25917(T)	98.5	-	-	-	-	-	-

			S. siphonella						
EA307	KY655408	*Pseudoalteromonas shioyasakiensis* SE3(T)	100	-	-	-	-	-	-
EA308	KY655409	*Vibrio harveyi* NBRC 15634(T)	100	-	-	-	-	-	-
EA309	KY655410	*Photobacterium angustum* ATCC 25915(T)	98.8	-	-	-	-	-	-
EA310	KY655411	*Vibrio owensii* LMG 25443(T)	99.9	-	-	-	-	-	-
EA311	KY655412	*Pseudoalteromonas shioyasakiensis* SE3(T)	100	-	-	-	-	-	-
EA312	KY655413	*Psychrobacter alimentarius* JG-100(T)	99.9	-	-	-	-	-	-
EA313	KY655414	*Pseudoalteromonas shioyasakiensis* SE3 (T)	98	-	-	-	-	-	-
EA314	KY655415	*Halomonas aquamarina* DSM 30161(T)	99.9	+++	++++	-	+	-	-
EA315	KY655416	*Vibrio neocaledonicus.*NC470(T)	99.9	-	-	-	-	-	-
EA316	KY655417	*Kocuria flava.*HO-9041(T)	99.8	-	-	-	-	-	-
EA317	KY655418	*Vibrio thalassae.*MD16(T)	98.8	-	-	-	-	-	-
EA318	KY655419	*Psychrobacter alimentarius* JG-100 (T)	99.7	-	-	-	-	-	-
EA319	KY655420	*Vibrio neocaledonicus* NC470 (T)	99.9			+	+	-	-
EA320	KY655421	*Vibrio campbellii.* CAIM 519 (T)	100	w	+++	+	-	-	-
EA321	KY655422	*Vibrio alginolyticus.*NBRC 15630 (T)	99.7	-	-		-	-	-
EA322	KY655423	*Psychrobacter alimentarius.*JG-100 (T)	99.9	-	-	+		+	+
EA323	KY655424	*Vibrio azureus.*NBRC 104587(T)	98.4	+	+++	-	-	-	-
EA324	KY655425	*Pseudoalteromonas shioyasakiensis* SE3(T)	99.3	++++	++++	-	+	-	-
EA325	KY655426	*Bacillus licheniformis.*ATCC14580 (T)	98	-	-	-	-	-	-
EA326	KY655427	*Photobacterium angustum.*ATCC25915(T)	99.1	-	-	-	-	-	-
EA327	KY655428	*Pseudoalteromonas shioyasakiensis* SE3(T)	99.7	-	-	-	-	-	-
EA328	KY655429	*Bacillus safensis.* FO-36b(T)	99.2	-	-	-	-	-	-
EA329	KY655430	*Psychrobacter aquaticus* CMS56(T)	99.9	++++	++++	-	+	-	-
EA330	KY655431	*Psychrobacter alimentarius* JG-100(T)	99.9	w	++	-	-	-	-
EA331	KY655432	*Halomonas sulfidaeris.*ATCC BAA-803(T)	95.7	++	+	-	+	+	
EA332	KY655433	*Carnobacterium inhibens subsp. Inhibens* DSM 13024(T)	99.7	++	+	-	+	+	+
EA333	KY655434	*Psychrobacter alimentarius.*JG-100 (T)	99.3	-	-	-	-	-	-
EA334	KY655435	*Bacillus subtilis subsp. Inaquosorum* KCTC 13429 (T)	100	+++++	++++	-	-	-	-
EA335	KY655436	*Psychrobacter alimentarius* JG-100 (T)	98.6	+	+	-	-	-	-
EA336	KY655437	*Psychrobacter aquaticus* CMS56(T)	99.9	-	-	-	-	-	-
EA337	KY655438	*Vibrio sagamiensis* LC2-047(T)	98.5	++	++++	-	-	++	-
EA338	KY655439	*Psychrobacter alimentarius* JG-100 (T)	99.8	++	++	-	-	+	-
EA339	KY655440	*Vibrio azureus.*NBRC 104587(T)	98.4	-	-	-	-	+	-
EA340	KY655441	*Psychrobacter alimentarius* JG-100(T)	99.9	-	-	-	-	-	-
EA341	KY655442	*Vibrio sagamiensis* LC2-047(T)	98.5	++	++++	-	-	-	-
EA342	KY655443	*Vibrio thalassae* MD16(T)	98.1	+	++	-	-	-	-
EA343	KY655444	*Vibrio thalassae* MD16(T)	97.9	-	-	+	-	+	
EA344	KY655445	*Psychrobacter aquaticus* CMS56 (T)	99.5	-	-	-	-	-	-

Note: [a]Identification based on partial 16S rRNA gene sequence analyses of all antagonistic bacteria. [b]% similarity with closely related type strain [c]Antagonistic activity of all bacteria isolated in this study. The activity was measured after 3-5 days incubation at 28°C by measuring the clear zone of mycelial growth inhibition: w; weak, -, Negative; +, 3 mm; ++, between 4 to 6 mm; +++, between 7 to 9 mm; ++++, between 10 to 12 +++++, between 13 to 15.

Some new and novel strains were also recovered from three marine sponges studied. Two strains *Pseudoalteromonas* sp. (EA262) and *Alteromonas* sp. (EA274) isolated from *P. vastifica*, were novel strain showing low 16S rRNA gene sequence similarities (93% to 94%) with respective type strains. Using 16S rRNA gene sequence data, neighbor Joining (NJ) phylogenetic trees were constructed for bacterial isolates of two

marine sponges (Figures 3a and 3b). Bootstrap values were high in all three phylogenetic trees. Bacterial isolates with antagonistic activity were recovered with high bootstraps values.

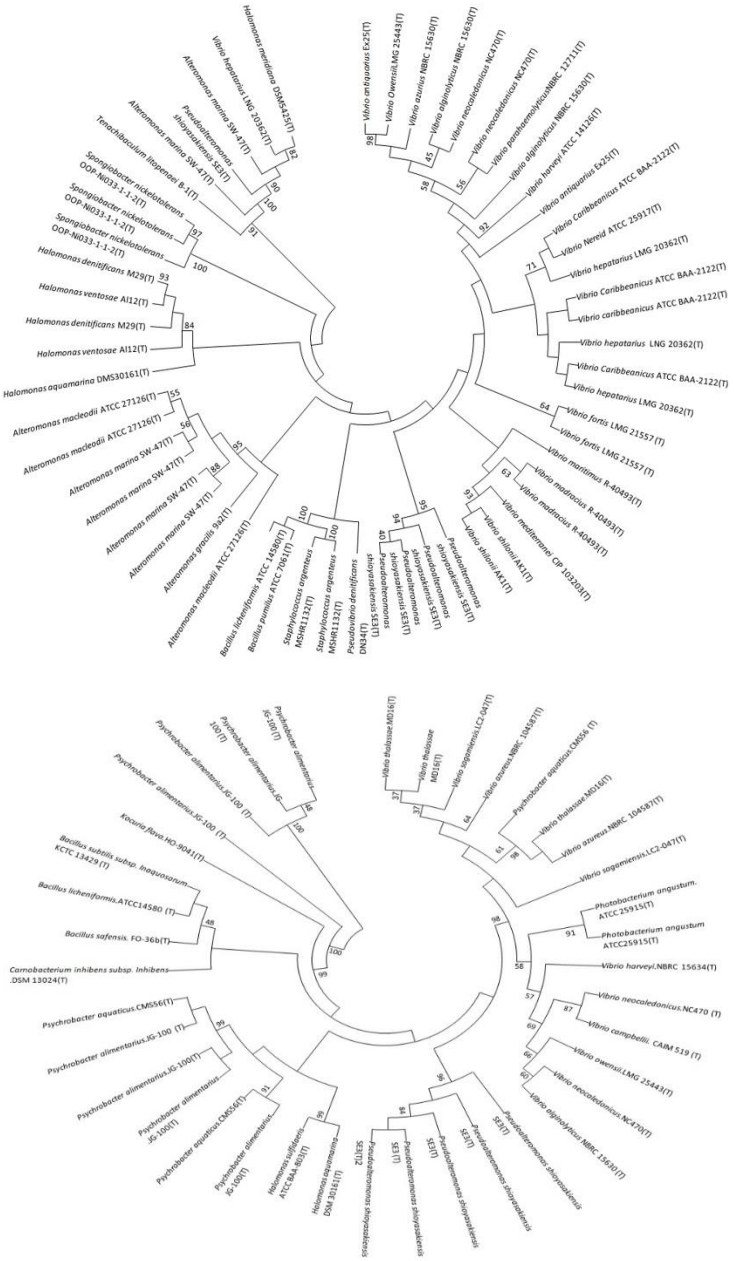

Figure 3. Phylogenetic distribution of bacteria isolated from two marine sponges (a) *Pione (Cliona) cf. vastifica,* and (b) *Siphonochalina siphonella* on the basis of 16S rRNA gene sequences obtained from bacteria and closely related sequences of the type strains of other species. The phylogenetic relationships were inferred from the 16S rRNA gene by using the neighbor-joining method from distances computed with the Jukes-Cantor algorithm. Bootstrap values (1,000 replicates) are shown next to the branches. GenBank accession numbers for each sequence are shown in parentheses. Bar, 0.01 accumulated changes per nucleotide.

Antagonistic activity

Bacteria isolated from marine sponges were screened for antagonistic activity against oomycetes, *Py. ultimum* and *P. capsici* and human pathogenic bacteria, *P. aeruginosa, S. aureus, E. coli* and *E. faecalis*. From sponge *P. vastifica,* only 24 (42%) from 57 bacterial strains were found to be antagonistic against both oomycetes. Antagonistic bacteria from *P. vastifica* belong to three major classes, *γ-Proteobacteria* (n=51; 89%), *Firmicutes* (n=5; 9%), and *Flavobacteria* (n=1; 2%). Dominant class of bacteria from *P. vastifica* was *γ-Proteobacteria*. Sponge *S. siphonella* exhibited presence of 13 (34%) from total 38 bacteria isolated. Antagonistic bacteria from this sponge belong to two major classes, *γ-Proteobacteria* (n=33; 87%) and *Firmicutes* (n=5; 13%) and dominat class was also *γ-Proteobacteria*. Bacterial strains from marine sponges were also screened for their antibacterial activity by using agar spot test. From sponge *P. vastifica,* twenty bacteria (35%) showed antibacterial activity. From these strains 4 strains were active against *P. aeruginosa,* 12 against *S. aureus,* 10 against *E. coli* and 3 against *E. faecalis*. From *P. vastifica,* one of the strain EA253 belong to *Staphylococcus* sp. showed antagonistic activity against oomycetes as well as against *P. aeruginosa*, *S. aureus,* and *E. coli*. Proportion of bacteria exhibiting antibacterial activity was high in *S. siphonella.* Table 2 showed 11 strains isolated from this sponge were active against different human pathogenic bacteria. Strain EA332 exhibited antifungal as well as antibacterial activity against *S. aureus, E. coli* and *E. faecalis*. In total 37 (39%) bacteria were antagonistic to oomycetes pathogens and 19 (20%) were antagonistic to human pathogenic bacteria. Dominant genus in this study from two marine sponges was *vibrio* as 42 different species of this genus was detected in two marine sponges.

Enzymatic activities of antagonistic bacteria

Bacteria isolated from marine sponges were evaluated for their ability for production of cell wall lytic enzymes. The isolated 124 bacterial strains were checked for protease, amylase, lipase and cellulase activities. Hydrolytic activities among the isolates from marine sponges are summarized in Table 3.

Table 3. Taxonomic identification, bacterial enzyme production on different enzymatic media used for culturing and further enzymatic activities.

			Enzymatic activity			
Lab no	**Accession Number**	**Similarity with closest type strain**	**Protease**	**Lipase**	**Amylase**	**Cellualse**
P. vastifica						
EA250	KY655351	*Vibrio hepatarius* LMG 20362(T)	-	-	-	-
EA251	KY655352	*Halomonas denitificans* M29(T)	-	-	-	-
EA252	KY655353	*Alteromonas marina* SW-47(T)	-	-	+	-
EA253	KY655354	*Staphylococcus argenteus* MSHR1132(T)	-	-	-	-
EA254	KY655355	*Pseudoalteromonas shioyasakiensis* SE3(T)	-	+	-	-
EA255	KY655356	*Halomonas denitificans* M29(T)	-	-	-	-
EA256	KY655357	*Vibrio fortis* LMG 21557 (T)	-	-	-	-
EA257	KY655358	*Vibrio harveyi* ATCC 14126(T)	-	-	-	-
EA258	KY655359	*Vibrio shilonii* AK1(T)	-	-	-	-
EA259	KY655360	*Vibrio parahaemolyticus*NBRC 12711(T)	-	+	+	-
EA260	KY655361	*Vibrio antiquarius* Ex25(T)	-	+	+	-
EA261	KY655362	*Tenachibaculum litopenaei* B-1(T)	-	-	-	-
EA262	KY655363	*Pseudoalteromonas shioyasakiensis* SE3(T)	-	+	-	-
EA263	KY655364	*Halomonas ventosae* Al12(T)	-	-	-	-
EA264	KY655365	*Pseudovibrio denitificans* DN34(T)	-	+	-	-
EA265	KY655366	*Halomonas ventosae* Al12(T)	-	+	-	-
EA266	KY655367	*Alteromonas marina* SW-47(T)	-	+	-	-

EA267	KY655368	*Halomonas aquamarina* DMS30161(T)	-	-	-	-
EA268	KY655369	*Alteromonas marina* SW-47(T)	-	+	+	
EA269	KY655370	*Vibrio Caribbeanicus* ATCC BAA-2122(T)	-	+	-	-
EA270	KY655371	*Vibrio hepatarius* LMG 20362(T)	-	+	-	-
EA271	KY655372	*Spongiobacter nickelotolerans* OOP-Ni033-1-1-2(T)	-	-	-	-
EA272	KY655373	*Pseudoalteromonas shioyasakiensis* SE3(T)		+		
EA273	KY655374	*Spongiobacter nickelotolerans* OOP-Ni033-1-1-2(T)	-	-	-	-
EA274	KY655375	*Alteromonas marina* SW-47(T)	-	+		
EA275	KY655376	*Alteromonas macleodii* ATCC 27126(T)	-	-	+	-
EA276	KY655377	*Spongiobacter nickelotolerans* OOP-Ni033-1-1-2(T)	-	-	-	-
EA277	KY655378	*Alteromonas marina* SW-47(T)	-	+	+	
EA278	KY655379	*Bacillus pumilus* ATCC 7061(T)	-	-	-	-
EA279	KY655380	*Vibrio antiquarius* Ex25(T)	-	+		-
EA280	KY655381	*Vibrio alginolyticus* NBRC 15630(T)	-	+	+	-
EA281	KY655382	*Vibrio Owensii*LMG 25443(T)	-	+		-
EA282	KY655383	*Vibrio maritimus* R-40493(T)	-	+	+	-
EA283	KY655384	*Vibrio neocaledonicus* NC470(T)	-	+	+	-
EA284	KY655385	*Vibrio hepatarius* LNG 20362(T)	-	-	-	-
EA285	KY655386	*Vibrio Caribbeanicus* ATCC BAA-2122*(T)*	-	-	-	-
EA286	KY655387	*Vibrio madracius* R-40493(T)	-	-	+	
EA287	KY655388	*Vibrio fortis* LMG 21557 (T)	-	-	-	-
EA288	KY655389	*Vibrio azurius* NBRC 15630(T)	-	-	-	-
EA289	KY655390	*Alteromonas gracilis* 9a2(T)	-	-	-	-
EA290	KY655391	*Vibrio madracius* R-40493(T)	-	-	-	-
EA291	KY655392	*Staphylococcus argenteus* MSHR1132(T)	-	-	-	-
EA292	KY655393	*Vibrio shilonii* AK1(T)	-	-	-	-
EA293	KY655394	*Alteromonas macleodii* ATCC 27126(T)	-	-	-	-
EA294	KY655395	*Staphylococcus argenteus* MSHR1132(T)	-	-	-	-
EA295	KY655396	*Vibrio hepatarius* LNG 20362(T)	-	-	-	-
EA296	KY655397	*Alteromonas macleodii* ATCC 27126(T)	-	-	-	-
EA297	KY655398	*Vibrio caribbeanicus* ATCC BAA-2122(T)	-	-	-	-
EA298	KY655399	*Halomonas meridiana* DSM5425(T)	-	-	-	-
EA299	KY655400	*Pseudoalteromonas shioyasakiensis* SE3(T)	-	-	-	-
EA300	KY655401	*Vibrio mediterranei* CIP 103203(T)	-	-	-	-
EA301	KY655402	*Bacillus lichemiformis* ATCC 14580(T)	-	-	-	-
EA302	KY655403	*Vibrio alginolyticus* NBRC 15630(T)	-	-	-	-
EA303	KY655404	*vibrio neocaledonicus* NC470(T)	-	-	-	-
EA304	KY655405	*Pseudoalteromonas shioyasakiensis* SE3(T)	-	-	-	-
EA305	KY655406	*Vibrio Caribbeanicus* ATCC BAA-2122(T)	-	-	-	-
EA306	KY655407	*Vibrio nereis* ATCC 25917(T)	-	-	-	-
S. Siphonella						
EA307	KY655408	*Pseudoalteromonas shioyasakiensis*.SE3(T)	-	-	-	-
EA308	KY655409	*Vibrio harveyi.*NBRC 15634(T)		+		

EA309	KY655410	*Photobacterium angustum.*ATCC 25915(T)	++	+		
EA310	KY655411	*Vibrio owensii.*LMG 25443(T)	-	-	-	-
EA311	KY655412	*Pseudoalteromonas shioyasakiensis.*SE3(T)	-	++	-	-
EA312	KY655413	*Psychrobacter alimentarius.*JG-100(T)	-	++	++	-
EA313	KY655414	*Pseudoalteromonas shioyasakiensis.*SE3 (T)	-	+	-	-
EA314	KY655415	*Halomonas aquamarina* DSM 30161(T)	-	+	-	-
EA315	KY655416	*Vibrio neocaledonicus.*NC470(T)	-	-	-	-
EA316	KY655417	*Kocuria flava.*HO-9041(T)	-	-	-	-
EA317	KY655418	*Vibrio thalassae.*MD16(T)		+		
EA318	KY655419	*Psychrobacter alimentarius.JG-100* (T)	-	-	-	-
EA319	KY655420	*Vibrio neocaledonicus.NC470* (T)	-	+	-	-
EA320	KY655421	*Vibrio campbellii.* CAIM 519 (T)	-	+	-	-
EA321	KY655422	*Vibrio alginolyticus.*NBRC 15630 (T)	-	+	-	-
EA322	KY655423	*Psychrobacter alimentarius.*JG-100 (T)	-	-	-	-
EA323	KY655424	*Vibrio azureus.*NBRC 104587(T)	-	+	-	-
EA324	KY655425	*Pseudoalteromonas shioyasakiensis.*SE3(T)	-	+	-	-
EA325	KY655426	*Bacillus licheniformis.*ATCC14580 (T)	-	++	+	-
EA326	KY655427	*Photobacterium angustum.*ATCC25915(T)	-	-	-	-
EA327	KY655428	*Pseudoalteromonas shioyasakiensis.*SE3(T)	-	-	-	-
EA328	KY655429	*Bacillus safensis.* FO-36b(T)	+	++	-	-
EA329	KY655430	*Psychrobacter aquaticus.*CMS56(T)	++	+	-	-
EA330	KY655431	*Psychrobacter alimentarius.*JG-100(T)	++	+	-	-
EA331	KY655432	*Halomonas sulfidaeris.*ATCC BAA-803(T)	++			
EA332	KY655433	*Carnobacterium inhibens subsp. Inhibens.*DSM 13024(T)	-	+	-	-
EA333	KY655434	*Psychrobacter alimentarius.JG-100 (T)*	-	++	-	-
EA334	KY655435	*Bacillus subtilis subsp. Inaquosorum.*KCTC 13429 (T)	+++	++	++	-
EA335	KY655436	*Psychrobacter alimentarius.*JG-100 (T)	-	+	-	-
EA336	KY655437	*Psychrobacter aquaticus.*CMS56(T)	-	-	-	-
EA337	KY655438	*Vibrio sagamiensis.*LC2-047(T)	-	-	-	-
EA338	KY655439	*Psychrobacter alimentarius.*JG-100 (T)	-	-	-	-
EA339	KY655440	*Vibrio azureus.*NBRC 104587(T)	-	-	-	-
EA340	KY655441	*Psychrobacter alimentarius.*JG-100(T)	-	-	-	-
EA341	KY655442	*Vibrio sagamiensis.*LC2-047(T)	++	++		
EA342	KY655443	*Vibrio thalassae.*MD16(T)	-	-	-	-
EA343	KY655444	*Vibrio thalassae.*MD16(T)	-	+	-	-
EA344	KY655445	*Psychrobacter aquaticus.*CMS56 (T)	-	-	-	-

(Note: Production of protease, amylase, lipase, and cellulase was determined by plate assay. Enzymatic activity was estimated as zone of halo formed around bacterial colonies: -, Negative; +, 3 mm; ++, between 4 to 5 mm; +++, between 6 and 7 mm.)

Isolates from *P. vastifica* showed activity for both lipase (n=18, 31%) and amylase (n=10, 17%) while no bacterial isolates displayed protease and cellulase activity. Number of bacteria producing hydrolyzing enzymes *S. siphonella* was comparatively high from other two marine sponges. Lipase producing bacteria was high (n=22, 58%) while protease (n=7, 18%) and amylase (n=3, 8%) producing bacteria were comparatively low while all isolates were negative for production of cellulase. Mostly *γ-Proteobacteria* and *Firmicutes* strains displayed high lipase and protease production. Only one strain isolated from *S. siphonella* belonging to *Bacillus sp.* (EA334) was positive for production of protease, lipase and amylase production while few strains were positive for production of one or two different enzymes. The number of bacteria exhibiting lipase activity (n=40; 42%) from two marine sponges was high as compared to other enzymatic activities.

DISCUSSION

Several previous studies have reported significance of microbial communities from marine sponges (Koopmans et al., 2009; Brinkmann et al., 2017). Therefore, sponges can be an untapped source of microbes that can be used as source of antibiotics and bioactive compounds. The objective of this study was to isolate bacteria, identify and screen them for their potential against different fungal and bacterial pathogens. We have used four different types of media for cultivation of bacteria and the high numbers of bacteria were recovered from ½ R2A and ½ TSA indicating that these two media contents are favorable for growth of bacteria from marine sponge. ½ R2A medium showed best recoverability as it contains nutrients in low concentration and sea water added as it contains different salts hence support growth of marine symbiotic bacteria. It was reported previously that low concentration of protein and nitrogen is important to recover diverse groups of bacteria (Joint et al., 2010; Medina et al., 2017).

In current study, the 16S rRNA sequences of bacteria associated with three marine sponges, *P. vastifica*, *S. siphonella* and *S. moliss* were analyzed phylogenetically. Bacterial isolates from these three marine sponges belong to 25 different genera and in turn assigned to five different classes i.e., *α-Proteobacteria,* □□-*Proteobacteria, Firmicutes, Flavobacteria* and Actinobacteria. Dominant class of bacteria was □-*Proteobacteria* and mostly bacteria belong to genus *Vibrio* where twenty-three different species were identified from total isolated bacteria (Table 2). *Vibrio* is a genus of marine bacteria comprising of 74 species and mostly commensal with Porifera sponges (Hoffmann et al., 2010). *γ-Protobacteria* in this study comprised of eight different genera i.e., *Vibrio*, *Pseudovibrio*, *Halomonas*, *Alteromonas*, *Pseudoalteromonas*, *Spongiobacter*, *Photobacterium* and *Psychrobacter*. It is previously known that from marine environment *γ-Protobacteria* produced the high number of bioactive metabolites (Long & Azam, 2001). Screening of bacterial isolates resulted in 56 potential strains capable to inhibit either fungal or bacterial pathogen. Highest numbers of antagonistic bacteria were recovered from marine sponge *P. vastifica*. Dominant class of bacteria was *γ-Protobacteria* comprising of 9 different genera where *vibrio* was dominant genus. In this study 27 different species of genus *vibrio* have been identified that is really interesting. Most of strains related to this genus showed inhibition either against pathogenic fungi or bacteria. No such studies including screening of bacterial isolates for antagonistic activities have been reported before from marine sponge *P. vastifica.* Only one study related to boring sponge, *P. vastifica* reported quorum-quenching (QQ) of bacterial isolates from marine sponge as well as QQ activity of sponge extract (Saurav et al., 2016). Many potential isolates were recorded for QQ activity as well as extract of sponge also showed QQ activity. Our study is first to describe diversity of culturbale bacteria from *P. vastifica* and their identification and screening for bioactive characteristics. *γ-Protobacteria* is most common cultivable group of bacteria associated with sponge or found in surrounding water (Taylor et al., 2007; Webster and Taylor, 2012). Several previous studies have reported dominance of *γ-Protobacteria* using high cultivation techniques (Montalvo et al., 2014; Esteves et al., 2016).

Biologically active compounds of marine *vibrios* have been reported as a rich source of novel biologically active metabolites (Oclarit et al., 1994; Chen et al., 2012). More than 90 different bioactive metabolites have been isolated from this class of bacteria and many were active against pathogenic bacteria. These antibacterial compound productions are important for these rhizopheric bacteria as their ecological role in sponges hence increase their abundance (Oclarit et al., 1994; Mansson et al., 2011). In sponge *P. vastifica* second dominant genus was *Alteromonas.* Species of *Alteromonas* are known to produce important secondary metabolites of clinical and ecological significance. A strain of *Alteromonas* associated with sponge *Halichondria okadai* produced a bioactive tetracyclic alkaloid showing both antimicrobial and cytotoxic activity (Shigemori et al., 1992). Another dominant genus in *S. siphonella* was *Psychrobacter* comprising of 11 different species. Previous studies report antibacterial activity of *Psychrobacter* but no antifungal activity found yet from this genus (Kennedy et al., 2010). Strain EA329 showing close 16S rRNA similarity to *Psychrobacter aquaticus* exhibited strong antifungal activity in our study that is first reported. Six bacterial strains from *P. vastifica* and only one strain from *S. siphonella* showed < 97% of 16S rRNA gene sequence similarity to closely related strains hence likelt to be new or novel new taxa.

Sponge-associated bacteria actively participate in hydrolysis of accumulated macromolecules inside sponge by using their extracellular enzymes. In this natural system different types of substrate are available and hydrolytic enzymes are released by bacteria to dissolve and absorb them. Nutrients released from these substrates are then utilized by sponges (Marx et al., 2007; Lee et al., 2001). In our study high percentage of lipase activity was detected in strains from both sponges. Lipases are vital biocatalysts and can be able to hydrolyze various types of substrates and are much stable in organic solvents (Karpushova et al., 2005). Amylase producing bacteria were also detected from both sponges. Amylase helps in degradation of starch molecules and has its uses in

pharmaceutical as well as in textile, cosmetic, and paper industries. Sponge-associated bacteria also produced other hydrolytic enzymes such as protease, cellulase and amylase suggests that these enzymes are important for degradation of organic substrate and regenerate nutrients in surrounding environment and sponge use them as food (Shanmughapriya et al., 2009). Most of the isolates exhibiting enzymatic activities were related to *γ-Protobacteria* and belonging to *vibrio* and *Psychrobacter*. In our study *vibrio* is dominant genus among culturable bacteria from both sponges showing antimicrobial and different enzymatic activities.

CONCLUSION

Our data reveals that what kind of important role sponge-associated heterotrophic bacteria are playing in seawater by producing antimicrobial compounds and degradation of substrate excreting hydrolytic enzymes. This work improves our knowledge about functional role of these bacteria in marine sponges from Red sea. Future studies related to their metabolite identification and enzyme studies will elucidate importance of novel and potential strains associated with sponges.

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