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Phylogenetic Investigations on the Endosymbiotic Bacteria of *Axinella donnani*

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Abstract

The present study focuses on the isolation of endosymbiotic bacteria from eight different types of sponges. The sponges were collected from the southern peninsular coast of India and identified as *Sigmadocia carnosa, Ircinia fasciculate, Callyspongia diffusa, Zygomycale angulosa, Clathria vulpine, Clathria gorgonids, Phloeodictyon species* and *Axinella donnani*. For the isolation of endosymbionts, the collected sponge species were cultured in three different media such as Nutrient agar media, Zobell marine agar and Zobell marine agar + sponge extracts. The sponge extracts supplemented media produced high bacterial growth than the other media. The sponge *A. donnani*, recorded the highest bacterial counts. Of which, 13 endosymbiotic bacterial strains (ESB) of *A. donnani* were screened against common pathogenic bacteria. The strains ESB-3 and ESB-7 were identified to be potential strains exhibiting significant antibacterial property. The antibacterial activity was evaluated through testing against various shrimp pathogens (*Vibrio esturiances, Vibrio alginolyticans, Vibrio harvae, Aeromonas hydrophila* and *Pseudomonas aerogenosa)* as well as human pathogens (*Streptococcus hemolyticus, Vibrio fisheri, Escherichia coli, Morgenella morgenii,* and *Bacillus cereus)*. The strains exhibited significant activity against all the shrimp and human pathogens. The unknown bacterial strain (ESB3 and ESB7) were identified using 16S rRNA gene technique to be *Bacillus subtilis,* Further, sequencing methodologies verified that the FASTA sequence of ESB3 contains 994 residues and that of ESB7 contains 1023 residues. The result so these findings are presented and elaborately discussed in the following paper.

Keywords: Nutrient agar media; Endosymbionts; Immunosuppressive; Shrimp

Introduction

Sponges (phylum Porifera) are the most primitive of the multi-celled animals, that have existed for about 700–800 million years. Approximately 15000 sponges have been described in the world and most of them live in marine waters, only 1% of the species inhabits freshwater [1]. According to Thomas (1998), India has more than 5000 species of marine sponges. But only 486 species has been described and reported. The reported landing species are *Callyspongia sp, Sigmadocia sp, Dendrilla nigra, Clathria gorgonoides* and *Axinella donnani*. All these species contained potent biologically active secondary metabolites [2].

A wide range of bioactive metabolites have been found in about 5000 from 500 marine sponges, most of them are reported to be bioactive. Up to 2014, five compounds or natural semisynthetic analogues which originate from the sponges have been resolved for medicinal purposes, and 13 compounds are in clinical trial, mostly as anticancer drugs, and 100 compounds are under preclinical trial [3]. Marine organism based biologically active natural drugs are used to treat many dangerous diseases and help improve our immune system [4].

However, an increasing role has been played by sponge associated microorganisms (endosymbionts) in the production of antibiotics and other drugs for the treatment of serious diseases. The identified bioactive substances from the endosymbionts showed therapeutic activity like anticancer, antibacterial, antifungal, antiviral, antiprotozoal, anthelmintic, anti-inflammatory, immunosuppressive and antifouling activities and the active compound are classified into the chemical groups such as peptides, polyketides, alkaloids, phenazines, isoprenoids, indolocarbazoles, sterols, fatty acids, and terpenes [5-8]. Without the necessity of harvesting or cultivation of the sponge, large amounts of metabolites can be produced [9].

In recent years, many bioactive compounds have been extracted from various marine organisms like seaweeds, sea grass, tunicates, sponges, soft corals, sea hares, nudi branches and bryozoans etc. Among the isolated potential metabolites, those from sponges are the most predominant [10]. In spite of the successes in drug discovery these associated microbes have received very little attention. The difficulty in the search of metabolites from sponge associated marine organism is mainly due to their non-cultivability [11]. It was estimated that 99% of sponge associated bacterial endosymbionts are uncultivable under laboratory condition using available media [12].

The sponge microbial association is a topic of research for a long time. Sponges are host organisms for various symbiotic microorganisms that include: archaea [13], bacteria [14], cyanobacteria [11], microalgae [15] and other sponges [16]. According to Vacelet and Donadey, the associated microorganisms may constitute up to 40% of sponge body mass [17]. Perusal of literature indicated the relation of sponges with bacteria. The relationship between the sponge and microorganisms are

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complex and complicated. The complexity of these microbes induced the microorganisms to synthesize effective metabolites with vast biodiversity [18].

In the present study sponges were collected from the southern peninsular coast of India. Studies involving the screening, isolation and characterization of bioactive compounds from marine bacteria on a systematic scale remains unencroached on. In the present study we have taken efforts to isolate and characterize the bioactive potential of marine bacteria associated with selected sponges The antibacterial activity of the endosymbionts was determined against 10 pathogenic bacteria of Human and Shrimp. Results found that the sponge *A. donnani* produced potent antibacterial activity than the other organisms. For the identification of the associated bacteria the 16SrRNA sequencing was performed. These findings suggested that the identified strains belonged to *Bacillus subtilis*. The evolutionary relationship associated with the sponge *A. donnani* using sequence data was generated based on 16S rRNA and comparisons made with the help of databases through the BLAST programme.

Materials and Methods

Collection and identification of sponges

The ecofriendly catch method was followed for the collection of marine sponges at different locations of southern peninsular coast of India (Arokyapuram, Muttom and vizhinjam). The collected specimens were identified with the help of Dr. P.A. Thomas, Sponge Taxonomist, Scientist (Retd), CMFRI, Vizhinjam. The color, code and the collected places of sponges were tabulated in Table 1.

Isolation of antibiotic producing endosymbiotic bacteria (APEB) from sponges

For the isolation of endosymbionts, the collected sponges were cultured in three different media such as Nutrient agar media, Zobell marine agar and Zobell marine agar + sponge extracts used as substrates. Initially, the separated sponge extracts were serially diluted with normal saline (NS) and streaked on the appropriate plates.

Quantitative analysis of endosymbionts

S. carnosa, I. fasciculate, C. diffusa, Z. angulosa, C. vulpina, C. gorgonides, P. sps and A.donnani (1 sq cm piece) were cultured in broth culture of nutrient agar + sponge extracts and Zobell marine agar + sponge extracts. The viable colonies were counted using pour plate method and the potent strain identified.

Antibacterial activity of endosymbionts

Test microorganisms: The antibacterial activity of the endosymbiont was determined against 10 pathogenic bacteria purchased from MTCC (Microbial Type culture collection), Chandigarh. The test organisms and their strain details are given in the Table 2.

Antibacterial assay: An agar-well diffusion method was employed for determination of antibacterial activities [19]. The Petri plates were prepared with 20 ml of sterile MHA. The plates were allowed to solidify for 5 minutes and the tested cultures were swabbed on top of the solidified media and allowed to dry for 10 min. Wells (4.6 mm in diameter) were cut from the agar with a sterile borer and 40 µl extract solutions were delivered into them. The extracts (well) were placed on the surface of medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 35°C for 24 hrs. Antibacterial activity was evaluated by measuring the diameter of inhibition zone. The zones of inhibition were measured in mm. Assay was carried out in triplicate and control plates were also maintained.

Sequencing and phylogenetic analysis of 16S rRNA gene

The DNA was extracted using the method as described by Chen and Kuo with slight modification and visualized on 1.0% agarose gel [20]. The 16S ribosomal DNA (rRNA) was amplified by PCR using 16SF: GAATCATATGCTGCGCCGTC and 16SR: ATCGGAACGCCATCCACTTC as primers. Thermal cycling was performed in an Applied BioSystem GeneAmp PCR system 2700 using Taq Polymerase (Fermentas) according to the following settings: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 1 min., 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 minutes. The PCR product after visualization was purified from the gel using PCR product purifying kit of Wizard SV Gel and PCR Cleanup System (Promega). The sequencing was performed by the DNA Sequencer ABI 3130 Genetic Analyzer (Applied BioSystems). The 16S rRNA sequence was compared to other prokaryotic 16S rRNA sequences by using the similarity search analysis service of NCBI (BLAST). For the construction of the phylogenetic tree and determination of the nearest database neighboring sequences, the sequences of isolates were aligned using CLUSTAL X program version 1.8 [21]. The sequences for the closest neighbors (approx. bp 1600) were used for the evolutionary study.

Results and Discussion

The South Peninsular coast was found to be an excellent area for the collection of marine sponges [22]. In, the present study, all the sponge species were collected from southern peninsular coast of India. The sponge species which were collected and used in the current study are shown in Figure 1. An earlier study by Sunil et al [23] showed that supplementing the media with sponge extract produced largest number of colonies in case of the sponge *Tethya crypta* associated microorganisms. The present study also clearly indicated that the sponge extract supplemented media produced more bacterial growth than the other media. Number of colonies produced on various media

S. No.	Sponge	Color	Code	Collected places
1	*Sigmadocia carnosa*	Brown	MS-1	Vizhinjam
2	*Ircinia fasciculate*	Brownish yellow	MS-2	Vizhinjam
3	*Callyspongia diffusa*	Yellow	MS-3	Vizhinjam
4	*Zygomycale angulosa*	Yellow	MS-4	Arokyapuram
5	*Clathria vulpina*	Brownish Yellow	MS-5	Arokyapuram
6	*Clathria gorgonids*	Reddish Yellow	MS-6	Muttom
7	*Phloeodictyon sp.*	Pink	MS-7	Muttom
8	*Axinella donnani*	Black	MS-8	Arokyapuram

Table 1: The collected sponges and their color, codes and collection spots.

S.No.	Bacteria	Gram stain
1	*Micrococcus luteus*	GRAM POSITIVE
2	*Bacillus cereus*	GRAM POSITIVE
3	*Bacillus subtilis*	GRAM POSITIVE
4	*Staphylococcus aureus*	GRAM POSITIVE
5	*Staphylococcus epidermidis*	GRAM POSITIVE
6	*Escherichia coli*	GRAM NEGATIVE
7	*Proteus vulgaris*	GRAM NEGATIVE
8	*Pseudomonas aeruginosa*	GRAM NEGATIVE
9	*Vibrio alginolyticus*	GRAM NEGATIVE
10	*Aliivibrio fischeri*	GRAM NEGATIVE

Table 2: Test organisms.

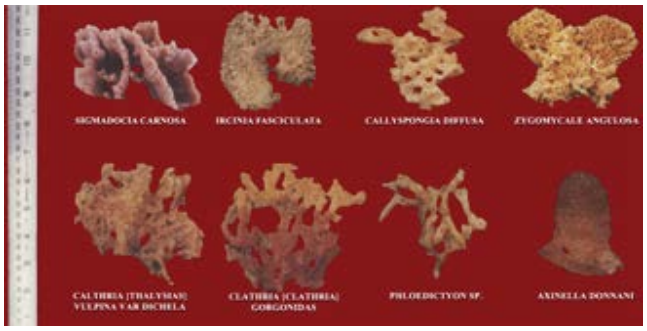


Figure 1: Sponge species.

containing antibiotic producing endosymbiotic bacteria were shown in Table 3.

Among the eight isolated organisms *S. carnosa, C. diffusa* and *A. donnani*, produced predominant numbers of bacterial colonies in all culture media compared to the rest. *Callyspongia diffusa* a total of 20 bacterial strains were isolated: 6 on nutrient agar, 6 on zobell marine and 8 on Marine extract supplemented-agar. Likewise the total of bacterial strains in *Sigmadocia carnosa* is 13: 2 on nutrient agar, 3 on zobell marine agar and 8 on Marine extract supplemented-agar and *A. donnani* produced 10 strains: 3 on nutrient agar, 3 on zobell marine agar and 4 on Marine extract supplemented-agar. The quantitative analysis reported that the number of colonies produced in 1 cm^2 area of sponge. The sponges *S. carnosa, C. diffusa* and *A. donnani* contained very thick bacterial population, while Nutrient agar + sponge extracts of both *C. diffusa* and *A. donnani* contained 9 and *S. carnosa* contained 7. Similarly Zobell marine agar + sponge extracts contained 9, 10 in *S. carnosa, C. diffusa* and *13 in A. donnani*. The sponges *S. carnosa, C. diffusa* and *A. donnani* contained very high bacterial growth as Nutrient agar + sponge extracts of both *C. diffusa* and *A. donnani* contained 9 and *S. carnosa* contained 7. Similarly Zobell marine agar + sponge extracts contain 9, 10 in *S. carnosa, C. diffusa* and *13 in A. donnani*. Among the three sponge species, *A. donnani* exhibited highest activity. Table 4 clearly indicated that the sponge extract supplemented media produced more bacterial growth than the other media.

The sponges *S. carnosa, C. diffusa* and *A. donnani* contained very thick bacterial population as Nutrient agar + sponge extracts. *C. diffusa* and *A. donnani* contain 9 and *S. carnosa* contain 7 colonies. Similarly Zobell marine agar + sponge extracts contain 9 in *S. carnosa,* 10 in *C. diffusa* and 13 in *A. donnani*. Among the three sponge species, *A. donnani* produced potent activity. The sponge-associated antogonistic actinomycetes were isolated from two marine sponges (*Dendrilla nigra and A. donnani*) reported for potent biological activity [24]. In *A. donnani*, the predominant numbers of bacterial colonies were noted in the culture media than the others. The endosymbiotic bacterial strain (ESB) of *A. donnani* was tested against 10 bacterial cultures is shown in Table 5 and 6.

Among the 13 tested organisms ESB-3 and ESB-7 showed highest activity. The Australian marine sponge, *Axinella sp*., possesses anti-bacterial activity against Helicobacter pylori [25]. The earlier report by Dhinakaran et al showed that the sponge associated strain of *Echinodictyum gorgonoides* was tested for its antibacterial activity against various human pathogens (*E. coli, Proteus Spp, S. aureus, Pseudomonas* and *B. subtilis*) and Annie et al [26] reported the antibacterial activity of *A. donnani* against fish pathogens (*Aeromonas hydrophila, Pseudomonas aerug-inosa, Vibrio alginolyticus, V. anguillarum, V.fischeri, V. fluvialis, V. pelagius,* and *V. vulnificus)* [27]. Many bacteria and cyanobacteria associated with sponges have been reported in the past to be the sources of antibiotics and other bioactive compounds in the marine environment reported a phenolic compound 2-(2,4-dib-romophenoxy)-4,6-dibromophenol was obtained from the sponge extracts of Dysidea granulose from Lakshadweep islands,

Sponge species	No. of colonies produced in various media			
	Nutrient agar	Zobell marine agar	Zobell marine agar + sponge extracts	Total
S. carnosa	2	3	8	13
I. fasciculate	2	3	4	9
C. diffusa	6	6	8	20
Z. angulosa	2	2	4	8
C. vulpina	2	2	5	9
C. gorgonids	2	2	3	7
P. sp.	2	2	4	8
A. donnani.	3	3	4	10

Table 3: Isolation of antibiotic producing endosymbiotic bacteria from sponges.

Sponge species	No. of colonies produced in 1c.m^2 area of sponge	
	Nutrient agar + sponge extracts	Zobell marine agar + sponge extracts
S. carnosa	7	9
I. fasciculate	5	5
C. diffusa	9	10
Z. angulosa	4	6
C. vulpine	5	6
C. gorgonids	6	5
P. sp.	6	8
A. donnani.	9	13

Table 4: Quantitative analysis of endosymbionts.

Bacteria	Antibacterial Activity									
	Gram Positive					Gram Negative				
	Micrococcus luteus	*Bacillus cereus*	*Bacillus subtilis*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Escherichia coli*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Vibrio alginolyticus*	*Aliivibrio fischeri*
ESB – 1	-	+	++	-	+++	++	+	-	+	++++
ESB – 2	++	++++	-	+++	-	-	++	+	+++	-
ESB – **3**	**+++**	**++**	**++**	**++**	**++**	**-**	**++++**	**-**	**+**	**++**
ESB – 4	-	++	+	-	+	+++	-	++	-	-
ESB – 5	+	-	+++	++	-	+	-	-	-	+
ESB – 6	++	+	-	-	+++	-	+	++++	-	-
ESB – 7	**++**	**++**	**++++**	**++**	**+**	**+**	**+**	**-**	**-**	**+**
ESB – 8	++++	-	+	+	-	++	-	+	+++	+
ESB - 9	-	+	++	++	-	-	+	++	-	+
ESB - 10	++	+	-	+	++	+++	-	-	+	-
ESB - 11	-	++	+	-	-	-	+	++	++	+++
ESB - 12	-	-	-	++++	+	+	++	+++	-	-
ESB - 13	++	++++	+	-	-	+	+++	++	-	+

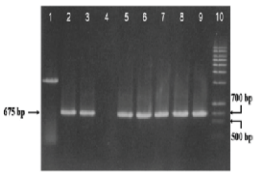
++++=30; +++=20-30mm; ++=10-20mm; +=1-10mm - = No Activity

Table 5: Antibacterial potential of endosymbiotic bacterial strain (ESB) of *A. donnani* against common pathogenic bacteria.

Pathogens	Bacterial Species	Zone diameter produced by ESB – 3 endosymbiont		Zone diameter produced by ESB – 7 endosymbiont	
		20°C	37°C	20°C	37°C
Shrimp pathogens	*V. esturiances*	++	+	+	+
	V. alginolyticans	++	-	+	-
	V. harvae	+	-	-	-
	A. hydrophila	++++	++	++	+
	P. aerogenosa	+++	+	+	-
Human pathogens	*S. hemolyticus*	++	-	+	-
	V. fisheri	+	-	-	-
	E. coli	+++	+	++	-
	M. morgenii	++	-	+	-
	B. cereus	+	-	-	-

++++=30; +++=20-30mm; ++=10-20mm; +=1-10mm - = No Activity

Table 6: The antibacterial activity of potent endosymbiont (ESB- 3 and ESB-7) against shrimp and human pathogens.



Lane 1 : 50 bp Molecular weight marker (promega)
Lane 2&3 : *Bacillus subtilis* strain marker
Lane 4 : Blank
Lane 5 &7 : ESB 3
Lane 6, 8 &9 : ESB 7
Lane 10 : Molecular weight marker (vivagen)

Figure 2: 16SrRNA sequencing.

>ESB 3
GGGTATAAACTGGAGGAGCCAAAAATGAATGAATAAATACCGTGTGCGCCTTTTTTCTGTATT
CGTTGTCTGTATGATTCTGGTGTTTTGTGTCCTCGGGCTGTTTTTACAGCAGCTTTTTGAAAC
ATCTGATCAAAGGAAAGCAGAGGAACACATTGAAAAAGAAGCAAAATACTTGGCTTCGCTTCT
TGATGCCGGCAATCTGAATGATCAAGCAAATGAGAAAATCATTAAAGATGCAGGCGGCGCGCT
TGATGTGAGCGACGGCAAGGTGCTGTGTCAACAACAGATAACAAGCTTTATTACGGACTTTCA
CTTAGAAGCGAAGGTGAAAAAACAGGATATGTGCTGCTTTCCGCCTCTGAAAAAAGCGACGGC
TTAAAAGGCGAATTGTGGGGGATGCTGACGGCCAGTCTTTGTACCGCATTTATCGTCATTGTT
TATTTTTATTCCAGCATGACCTCACGTTATAAAAGGTCAATTGAGTCAGCCACAAATGTAGCC
ACAGAACTCTCTAAGGGCAACTACGATGCACGGACATACGGCGGCTATATTAGGCGTTCTGAT
AAGCTAGGACATGCGATGAACAGCCTTGTATGCATTGAAAACATTGGCTCCGGGCTGATTATG
ATCGACGGAAGAGGCTTTATTAATCTCGTAAACAGGTCTTACGCCAAGCAGTTTCATATCAAT
CCGAATCATATGCTGCGCCGTCTTTATCACGATGCATTTGAACATGAAGAAGTGATCCAGCTT
GTCGAAGACATTTTTATGACGGAGACAAAGAAATGCAAGCTGTTAAGACTTCCGATCAAAATA
GAACGGCGTTATTTTGAAGTGGATGGCGTTCCGATTATGGGGCCGGACGATGAATGGAAAGGA
ATTGTGCTCGTTTTTCATGACATGACGGAAACAAAGAAATTAGAGCAGATGAGGAAGGATTTT
GTGGCCAATGTTTCTCATGAGCTGAAAACGCCGATTACGTCATAAAAGG

>ESB 7
GGGTATAAATTAGAGGAGCCAAAAATGAATGAATAAATACCGTGTGCGCCTTTTTTCTGTATT
CGTTGTCTGTATGATTCTGGTGTTTTGTGTCCTCGGGCTGTTTTTACAGCAGCTTTTTGAAAC
ATCTGATCAAAGGAAAGCAGAGGAACACATTGAAAAAGAAGCAAAATACTTGGCTTCGCTTCT
TGATGCCGGCAATCTGAATGATCAAGCAAATGAGAAAATCATTAAAGATGCAGGCGGCGCGCT
TGATGTGAGCGCATCCGTTATCGATACTGTCAACAACAGATAACAAGCTTTATTACGGACTTT
CACTTAGAAGCGAAGGTGAAAAAACAGGATATGTGCTGCTTTCCGCCTCTGAAAAAAGCGACG
GCTTAAAAGGCGAATTGTGGGGGATGCTGACGGCCAGTCTTTGTACCGCATTTATCGTCATTG
TTTATTTTTATTCCAGCATGACCTCACGTTATAAAAGGTCAATTGAGTCAGCCACAAATGTAG
CCACAGAACTCTCTAAGGGCAACTACGATGCACGGACATACGGCGGCTATATTAGGCGTTCTG
ATAAGCTAGGACATGCGATGAACAGCCTTGAGACGGCAAGGTGCTGTATGGGTCAAACGGGAG
AGGATCGGCTGCTGACAGTCATTGAAAACATTGGCTCCTTATGATCGACGGAAGAGGCTTTAT
TAATCTCGTAAACAGGTCTTACGCCAAGCAGTTTCATATCAATCCGAATCATATGCTGCGCCG
TCTTTATCACGATGCATTTGAACATGAAGAAGTGATCCAGCTTGTCGAAGACATTTTTATGAC
GGAGACAAAGAAATGCAAGCTGTTAAGACTTCCGATCAAAATAGAACGGCGTTATTTTGAAGT
GGATGGCGTTCCGATTATGGGGCCGGACGATGAATGGAAAGGAATTGTGCTCGTTTTTCATGA
CATGACGGAAACAAAGAAATTAGAGCAGATGAGGAAGGATTTTGTGGCCAATGTTTCTCATGA
GCTGAAAACGCCGATTACATCAATAAAAGG

Figure 3: FASTA sequence of ESB3 and ESB7.

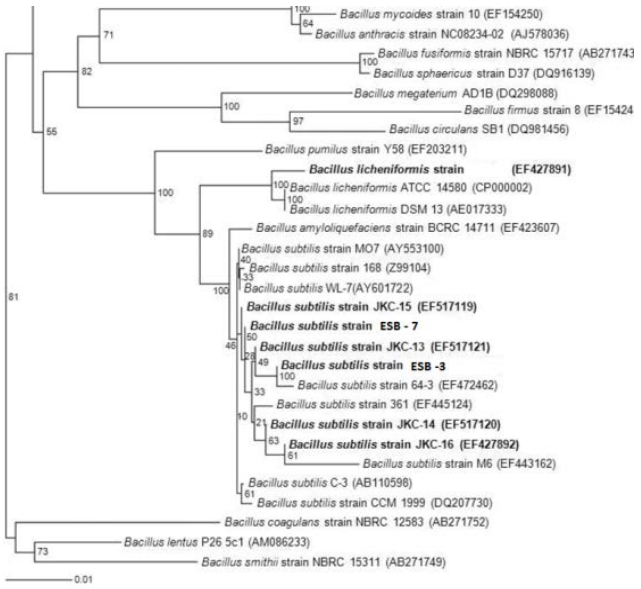


Figure 4: Phylogenetic analysis of ESB3 and ESB7.

against strains of MRSA and VRE [28]. In another study presence of phenolic compounds was confirmed by thin layer chroma-tography and associated Rf values of crude extract showed that the secondary metabolite was produced by endosymbiotic bacteria A. calcoaceticus in *D. granulosa* and was not from the sponge tissue [29]. Thus, the 2-(2, 4 -dibromophenoxy)-4,6-dibromo-phenol may be produced by the endosymbiotic bacteria of *D. granulose*. The suspected bioactive compounds include chemical entities such as peptides, polyketides, alkaloids, phenazines, isoprenoids, indolocarbazoles, sterols, fatty acids, and terpenes [5-8]. In the current study, although we have no conclusive evidence towards narrowing down on specifying any one such bioactive compound behind the observed antimicrobial activity, more studies are required in this direction for the isolation of these bioactive compounds. The present study also strongly supported the existing studies that *A. donnani* showed broad spectrum antimicrobial activity against 10 Human and Shrimp pathogenic bacteria.

16 S rRNA

Phylogenetic analysis based on 16SrRNA fragment is useful for understanding the basic relationship among strains [30]. The unknown strain of antagonistic bacteria associated with the sponge *A. donnani* is *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* [31]. Figure 2 represented the partial sequencing of 16SrRNA. It was reported that the unknown bacterial strains (ESB-3 and ESB-7) of the sponge *A. donnani* is *Bacillus subtilis.* The FASTA sequence of ESB3 contains 994 residues and ESB7 contains 1023 residues shown in Figure 3. The tree was constructed with the neighbor-joining method shown in Figure 4. Numbers on nodes indicate Bayesian posterior probability values. Genetic distances were computed by Kimura's two-parameter model. The 16S rRNA demonstrated that the identification of the unknown bacteria based on the ESB3 and ESB7 results pointed to *Bacillus subtilis.*

Conclusion

Eight different types of sponge species were collected from southern peninsular coast of India were found to high bacterial growth in sponge extract supplemented media. Among the eight isolated organisms *S.*

carnosa, C. diffusa and *A. donnani* produced predominant numbers of bacterial colonies in all culture media. Comparatively, *A. donnani,* showed highest antimicrobial activity than the other 3 species. Based on the identification of potent endosymbiotic bacterial strain (ESB) of *A. donnani* against 13 common pathogenic bacteria ESB-3 and ESB-7 were found to exhibit highest activity. The antibacterial activity of the potent endosymbiont (ESB- 3 and ESB-7) was determined against 5 shrimp and 5 human pathogenic bacteria. Further, the 16S rRNA partial sequencing reported that the unknown bacterial strains of ESB3 and ESB7 was *Bacillus subtilis.* The FASTA format sequences were used to construct the phylogenetic tree and it clearly indicated the evolutionary relationship between the species.

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