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, Zygosphaera angulosa, Clathria vulpina, Clathria gorgonides, 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 higher 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 esturianes, Vibrio alginolyticans, Vibrio harvey, Aeromonas hydrophila and Pseudomonas aerogena) 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 multicelled 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 gorgonides 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, antihelminthic, anti-inflammatory, immunosuppressive and antifouling activities and the active compound are classified into the chemical groups such as peptides, polyketides, alkaloids, phenazines, isopenoids, 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 (APEG) 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. 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 16SrRNA 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: GAATCATATGCTGGCCGCTG and 16SR: ATCGGAACGCCATCCATCCTGC 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 purification was used for sequencing using the gel with PCR product purification 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

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

| S. No. | Sponge | Color   | Code | Collected places |
|-------|--------|---------|------|------------------|
| 1     | Sigmadiscus carnosa | Brown | MS-1 | Vizhinjam |
| 2     | Ircinia fasciculata | Brownish yellow | MS-2 | Vizhinjam |
| 3     | Calypsigonia 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 2:** Test organisms.

| 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    | Alivibrio fischeri | GRAM NEGATIVE |
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 agar and 8 on Marine extract supplemented-agar. Likewise the total of bacterial strains in Sigm nodia 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 cm² 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 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 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 antigenic actinomyces 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 antibacterial 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 aeruginosa, Vibrio 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-romophenxy)-4,6-dibromophenol was obtained from the sponge extracts of Dysidea granulose from Lakshadweep islands.
against strains of MRSA and VRE [28]. In another study presence of phenolic compounds was confirmed by thin layer chromatography 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. granulosa*. 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 16S rRNA 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 16S rRNA. 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 *A. donnani* showed broad spectrum antimicrobial activity against 10 Human and Shrimp pathogenic bacteria.

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

| 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. harvei*                |                   | +     | -    | -     | -    |
| *A. hydrophila*            |                   | ++++  | +    | ++    | +    |
| *P. aerogenosa*            |                   | ++++  | +    | +     | -    |
|                            |                   |       |      |       |      |
| Human pathogens            |                   |       |      |       |      |
| *S. hemolyticus*           |                   | ++    | -    | -     | -    |
| *V. fisheri*               |                   | +     | -    | -     | -    |
| *E. coli*                  |                   | ++++  | +    | ++    | +    |
| *M. morganii*              |                   | ++    | +    | +     | +    |
| *B. cereus*                |                   | +     | -    | -     | -    |

++++=30; +++=20-30mm; +++=10-20mm; ++=1-10mm - = No Activity
carnosa, C. diffusa and A. donmani produced predominant numbers of bacterial colonies in all culture media. Comparatively, A. donmani showed highest antimicrobial activity than the other 3 species. Based on the identification of potent endosymbiotic bacterial strain (ESB) of A. donmani 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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