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Antibacterial potential of sponge endosymbiont marine *Enterobacter* sp at Kavaratti Island, Lakshadweep archipelago

Mohan Gopi[1,2*], Subramanian Kumaran[1*], Thipparamalai Thangappanpillai Ajith Kumar[1], Balaraman Deivasigamani[1], Kumarappan Alagappan[1], Srinivasan Guru Prasad[1]

[1]*CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai- 608 502, Tamil Nadu, India*
[2]*CMLRE Field Research Station, Agatti Island, Lakshadweep- 682 553, India*

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ABSTRACT

Objective: To isolate antibacterial potential of sponge endosymbiotic bacteria from marine sponges at Lakshadweep archipelago. Also to identify the potent bacteria by 16s rDNA sequencing and determine the antibacterial activity against clinical pathogens by MIC. **Methods:** Sponge samples was collected from sub-tidal habitats at Kavaratti Island and identified. The endosymbiotic bacteria were isolated and selected potential bacteria which show antibacterial activity in preliminary screening against clinical pathogens *Staphylococcus aureus (S. aureus)*, *Escherichia coli (E. coli)*, *Salmonella typhi (S. typhi)*, *Klebsiella pneumoniea (K. pneumoniea)* and *Streptococcus* sp. by disc diffusion assay. The crude extracts of potential bacteria LB3 was tested against clinical pathogens by MIC. The LB3 strain was identified by 16s rDNA sequencing, 1 111 bp was submitted in NCBI (HQ589912) and constructed phylogenetic tree. **Results:** Sponge sample was identified as *Dysidea granulosa (D. granulosa)* and potential bacteria LB3 identified as *Enterobacter* sp TTAG. Preliminary screening of sponge isolates against clinical pathogens, LB3 strain was selected as potential producer of secondary metabolites and crude extract was implies on MIC of LB3 have confirmed with lowest concentration of 5.0 mg/mL in broth medium influence of crude extract on growth inhibitory activity after 5 h of incubation period and completed the inhibitory activity at 15 h. **Conclusions:** The present study concluded that phylogenetic analysis of endosymbiotic bacteria *Enterobacter* sp from sponge *D. granulosa* of Lakshadweep islands showed significant antibacterial activity against clinical bacterial pathogens.

1. Introduction

During the last two decade, several studies have been performed to establish biologically active products from marine sources, which mostly deals with marine bacteria and their potential role in the production of metabolites is becoming thrust area for research, Sponges (Porifera) have been recognized as a rich source of novel compounds that are of potential interest to mankind[1]. Marine sponges are conspicuous members of the marine benthos, occurring worldwide from polar and temperate to tropical seas, from the intertidal to deep-sea environments[2]. The development of easy and economically feasible synthesis of bioactive compounds has been preferred to date by drug companies and many marine leads have not advanced in development after their chemical descriptions in the literature[3]. In recent years, our research have been focused on the isolation of cytotoxic molecules from marine sources mainly sponges having active secondary metabolites against broad spectrum of clinical and marine pathogens. Sponges are filter feeders, which live in areas with strong currents or wave action. Most carnivorous animals avoid sponges because of the splinter-like spicules and toxic chemicals produced/sequestered by the sponge. Sponges are an important component of the benthic fauna throughout the temperate, tropical, and polar habitats[4].

Sponges with enormous diversity of microorganisms have an explicit source of pharmaceutical valued products. The biology of the bacteria-sponge relationship has been elicited considerable interest among researchers to investigate marine organisms as sources of natural products[5]. The route filter feeding offers the possibility of associated bacteria to produce high amounts of

*Corresponding author: Mohan Gopi, CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai- 608 502, Tamil Nadu, India.
E-mail: gpsakthi@gmail.com; kumarun23@gmail.com

biomass, biological active secondary metabolites. These metabolites can easily be produced in large amounts on a biotechnological scale without the necessity to harvest or cultivate the sponge[6]. Thus an alternative strategy targeting the endosymbiotic bacteria of sponges for the screening of bioactive natural products may prove to be an effective approach to come to blows emerging multi resistant clinical pathogens.

The Union Territory of Lakshadweep is an archipelago situated in the Arabian Sea between 08° 00′ N and 12° 30′ N latitude and 71° 00′ E and 74° 00′ E longitude and at a distance of 220-440 km from the west coast of India. The islands have a lagoon area of about 4000 sq. km, territorial waters covering 20 000 sq. km, continental shelf of 4 000 sq. km and an EEZ of 0.4 million sq. km. Hitherto, the reports on the sponges recorded in the Lakshadweep atolls was a total of 91 species referable to 30 families and 66 genera[7]. Therefore, the present study was targeted to isolate the endosymbiotic microorganisms from sponges and to evaluate their antagonism against clinical pathogens. The present investigation is the second of its kind to phylogenetic study of endosymbiotic microorganisms in the Lakshadweep sea sponges.

2. Materials and methods

2.1. Collection and identification of sponges

Sample was collected by snorkeling and skin diving from the sub-tidal habitats at depths at 3 meter at different location of Kavaratti (Lat. 10° 33′ N, Long. 72° 38′ E) islands. The samples were placed inside sterile ethyl polythene bags at the underwater itself and transferred to the laboratory, where they were examined and processed to isolate bacteria. The sponge material was preserved in 70% methanol for identification purpose. Identification was carried out with the help of Dr. P. A. Thomas, Sponge Taxonomist, Scientist (Retd), CMFRI, Vizhinjam, India.

2.2. Isolation of sponge associated bacteria

Sponge sample were washed with jets of filtered and autoclaved sea water until they were visibly free from debris. Followed, the sponge surface was sterilized by a rapid wash of 70% ethanol and immediately immersed in sterilized seawater and then aspirated. One gram of sponge tissue was removed with a sterile scalpel and the tissue was immediately transferred to 99 mL sponge dissociation medium (2.7% NaCl, 0.008% KCl, 0.01% Na_2SO_4, pH 8). The samples are soaked for 20 min and then the tissue and diluents were macerated and the homogenate was spread on Zobell Marine agar 2216 (Himedia, Mumbai), by 50% sea water, using a dilution series of 10^{-5}. The plates were incubated at room temperature (approx. 27-30 ℃) for 3 d. The isolated colonies were repeatedly streaked to obtain pure cultures and stored in Zobell agar slants at 4 ℃ for further studies[8].

2.3. Bacterial extracts

Totally 7 sponge bacterial isolates (LB1 to LB7) were inoculated in each flask with 100 mL Zobell marine broth (Himedia, Mumbai) containing 500 mL Erlenmeyer flask in a shaker (30 ℃/250 rpm) for 48-74 h and the cells were separated by centrifugation (4 ℃/7 000 rpm/10 min) and supernatant was extracted using same volume of ethyl acetate, methanol, hexane and the solvent was removed at 37 ℃.

2.4. Preliminary screening for antibacterial activity

The five human pathogenic bacteria namely *Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Salmonella typhi (S. typhi), Klebsiella pneumoniea (K. pneumoniea)* and *Streptococcus* sp. were used in this study were obtained from Raja Muthaiya medical college, Annamalai University, Tamil Nadu. The human pathogenic bacterial strains were grown in 50 mL of nutrient broth at 37 ℃ and maintained in nutrient agar slant at 4 ℃. An overnight suspension culture of the five bacterial strains was spread on the Mueller-Hinton agar (MHA) media. Sterile discs were prepared and placed on the culture spread agar media. The discs were impregnated with sponge isolated bacterial extract and the commercial antibiotic chloramphenicol was included as a positive control to determine the sensitivity of the bacterial strains. The inoculated plates were incubated at 37 ℃ for 24 h. The antibacterial activity was evaluated by measuring diameter of the inhibition zone around the disc.

2.5. Characterization of potential LB3 isolate

2.5.1. Phenotypic Characterization

Potential isolate of sponge associated bacteria LB3 were showed excellent activity against fish pathogens. The potential bacterial isolates were cultured on agar medium for 24 h at 28 ℃, and its physiological and biochemical examination were carried out. Followed, the colony morphology, Gram-stained and motility was determined. The biochemical characters of potential bacteria was characterized with Biochemical kit-KB002 (Hi media, Mumbai) which includes Indole, Methyl red, Voges Proskeur′s, Citrate utilization, H_2S production and sugar utilization tests.

2.5.2. DNA isolation and 16S rDNA gene amplification

The potential bacteria LB3 were cultured in LB broth (Hi Media, Mumbai) for 2 d and total genomic DNA was extracted. The 16S rDNA sequences were amplified by polymerase chain reaction (PCR) using universal primers 8f (3′-A GAGTTTGATCCTGTGCTCAG-5′) and 1 490r (5′-GACTTACCAGGGTATCTAATCC-3′). PCR procedures were followed by the method of Kumaran *et al*[9].

2.5.3. Sequencing and phylogenetic analysis

The amplified PCR products were purified using a Genei PCR purification kit (Genei, Bangalore). Nearly full-full length sequences of the amplified 16S rDNA genes (1 510 bp) were obtained by automated sequencer (Bioserve Biotechnologies pvt. Limited, India). The sequences were edited by using Clustal X mega software and a BLAST search was performed in the National Center for Biotechnology Information (NCBI) database to identify the nearest neighbor

of the amplified sequence. The results of the sequencing were used for homology searches. Phylogenetic trees were inferred using the neighbor-joining method.

2.5.4.Nucleotide sequences accession number

The partially complete (1 111 bp) 16S rDNA sequences of *Enterobacter* isolate have been deposited in the GenBank database under accession number HQ589912.

2.6. Determination of minimum inhibitory concentration

In order to determining the *Enterobacter* sp TTAG for minimum inhibitory concentration (MIC) extract (2.5, 5.0, 10.0, 20.0, 30.0, 40.0 and 50.0 mg/mL) was prepared. These dilutions were added to tubes containing 1 mL Muller Hinton broth (Himedia, Mumbai) and 30 mL of bacterial suspension was also added. The tubes were incubated at 37 ℃ for 24 h. The MIC of the extract was determined for the most sensitive bacterial species. The lowest concentration of crude extract in broth medium that had inhibited the growth of the test microorganism was considered as MIC. Influence of crude extract on the growth inhibitory activity of LB3 against clinical pathogens and the OD (600 nm) was measured at an every hour interval compared with positive control without crude extract.

3. Results

Taxonomy of sponge sample collected from Kavaratti island, Lakshadweep archipelago was *Dysidea granulosa* (*D. granulosa*) (Order: Dendroceratida, Family: Dysideidae) (Figure 1). In the present study, seven different types of bacterial colonies were isolated from *D. granulosa* central part of the organic and/or inorganic skeleton of sponge body and they were separated by based upon the colony morphology (shape, size and colour). Total count ranged from a highest of 2×10^5 to a lowest of 3×10^3 CFU per gram of sponge tissue. A physiological and biochemical characteristic of potential bacteria LB3 was Gram-negative rod, motile, Vp, citrate positive and sugars were fermented on glycerol, sucrose, raffinose and sorbitol. Oxidase, indole, Mr, H_2S and urease showed negative.

Figure 1. Sponge sample *D. granulosa* isolated from Agatti island.

Preliminary screening of bacterial extracts by disc diffusion assay LB3 host sponge *D. granulosa* have confirmed significant antibacterial activity strain was selected as potential bacteria to produce active secondary metabolites against clinical pathogens were *S. aureus*, *E. coli*, *S. typhi*, *K. pneumoniea* and *Streptococcus* sp. The zone of inhibition was compared to positive control chloramphenicol and secondary metabolites of LB3 which more or less similar to commercial antibiotic (Table 1). Crude extract obtained with ethyl acetate, methanol and hexane from extracellular product of isolated bacteria LB3 showed significant antibacterial activity by disc diffusion assay using 500, 1 000 and 1 500 μg/mL of crude extract and the minimum concentration of 1 000 μg/mL shows maximum zone of inhibition against tested pathogens. The strain LB3 was identified by PCR amplification of 16S rRNA gene followed by the sequencing. The genomic DNA of the bacterial strain LB3 was isolated and confirmed by agarose gel electrophoresis. The 16S rRNA gene was amplified from the genomic DNA of the strain LB3 under the optimal conditions. Blast analysis of the sequence data revealed most identity with *K. pneumoniae*-v-N (GenBank accession number EU360116). Molecular identification of the 16S rRNA gene shows 99% identity at 100% coverage with *Enterobacter* sp. The sequenced 16S rRNA gene of strain (GenBank Accession number HQ589912) that was identified as 1 111 bp in length, and exhibited high similarity (99%) with the 16S rRNA gene of *Enterobacter* sp TTAG from GenBank database. Based on the 16S rRNA gene, a phylogenetic tree was generated (Figure 2). Minimum inhibitory concentration of *Enterobacter* sp TTAG have confirmed with lowest concentration of 5.0 mg/mL crude extract in broth medium against tested all clinical pathogens (Figure 3) after 5 h of incubation period.

Table 1
Determination of antibacterial activity of crude metabolites by disc diffusion method.

Clinical pathogens	Potential bacteria (LB3) inhibition zone in mm diameter
S. aureus	22
E. coli	25
S. typhi	19
K. pneumoniea	22
Streptococcus sp.	23
Positive control chloramphenicol	27

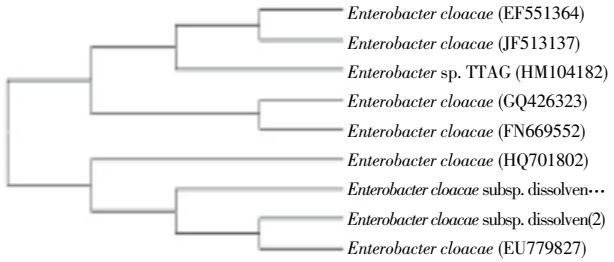

Figure 2. Phylogenetic tree constructed through Kimura 2-parameter model using the Neighborhood-joining method. Queried sequence: *Enterobacter* sp. TTAG(HM104182).

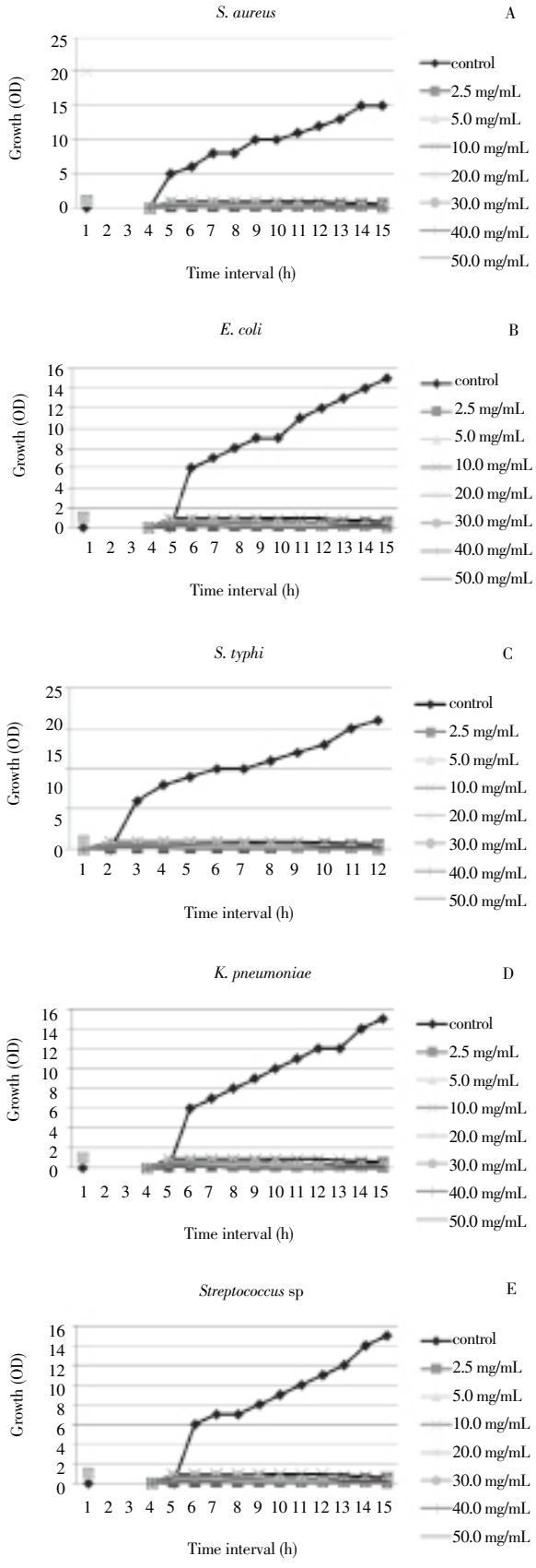

Figure 3. Effect of crude extract against clinical pathogens was *S. aureus*, *E. coli*, *S. typhi*, *K. pneumoniea* and *Streptococcus* sp measured after 5 h of incubation by OD at 600 (nm) at an every hour interval.

4. Discussion

Sponges are the oldest multicellular invertebrates, and they filter large quantities of seawater through their channel system. Bacteria, algae, phytoplankton, and fungi are the major components of the natural diet of sponges. Sponges are well known to harbor diverse microorganisms, which can contribute up to more than 40% of the sponge body volume exceeding microorganisms in seawater by two to four orders of magnitude[10]. The cultivable sponge-associated microbial diversity remains very unclear because of the difficulty in sponge-associated microbial isolation and pure cultivation. The current investigation of cultivable sponge-associated microbial diversity is mainly based on pure cultivation, single strain is isolated and cultured individually. The secondary metabolites secreted by endosymbionts were presumed to be accumulated in sponge tissue. In this study bacteria was isolated from sponges have been found to exhibit antibacterial activity meanwhile metabolites of sponge-associated bacteria have been determined.

The secondary metabolites of host sponge *D. granulosa* have confirmed significant antimicrobial activity and inhibited the growth of clinical pathogens. As earlier reported by[5] seventy-five marine bacterial strains associated with four species of sponges (*Echinodictyum* sp., *Spongia* sp., *Sigmadocia fibulatua* and *Mycale mannarensis*), agar-overlay method was used to screen for antibiotic production. Comparative to other sponge isolates, the LB3 strain inhibited the growth of all tested clinical pathogens assayed inducing inhibition of 25 mm in preliminary results. Some evidence indicates that many compounds previously found in sponges are biosynthesized through microorganisms associated with them or indeed produced by microorganisms[11]. Antibiotic production is subject to complex regulatory networks and is generally induced in stationary phase under conditions of nutrient limitation (Martin and Liras 1989). Antibacterials are thought to confer a selective advantage, when in competition with other bacteria populating for their survival in the same ecological niche. Minimum inhibitory concentration of LB3 was assayed against tested clinical pathogens have confirmed with lowest concentration of 5.0 mg/mL in broth medium influence of crude extract on growth inhibitory activity after 5 h of incubation period and completed the inhibitory activity at 15 h.

Shridhar *et al*[12-16] reported a phenolic compound 2-(2′,4′-dibromophenoxy)-4,6-dibromophenol was obtained from the sponge extracts of *D. granulosa* from Lakshadweep islands, against strains of MRSA and VRE. The secondary metabolite was produced by endosymbiotic bacteria *Enterobacter* sp in *D. granulosa* not extracted from sponge tissue. Thus, the 2-(2′,4′-dibromophenoxy)-4,6-dibromophenol may be produced by the endosymbiotic bacteria of *D. granulosa*. So instead of harvesting the whole sponge we could be producing the potential compound by culturing the microbe against fish pathogens. There is

now considerable evidence for the presence of a diverse assemblage of *Enterobacter* sp in the marine environment. Hentschel *et al*[17] reported the antimicrobial activities of marine sponges *Aplysina aerophoba* and *A. cavernicola* with phylogenetic analysis. The isolated bacteria showed conspicuous results against clinical pathogens of (*S. aureus, E. coli, S. typhi, K. pneumoniea* and *streptococcus* sp.). To our knowledge, this is the first time that a strain recovered genus Enterobacter sp has been isolated and cultured from Lakshadweep sea sponge. The 16S rRNA gene sequence of isolate LB3 shares 99% homology with that of *K. pneumoniae*, and is thus phylogenetically closely related to this species. *In vitro* antimicrobial screening of sponge crude extract showed antimicrobial activities against selected clinical isolates of bacteria and fungi[18]. In this regard, the present study implies that systemic drug development could be sustained using the associated endosymbionts instead of host sponges as an alternative of harvesting whole sponge. The endosymbiotic microorganisms could be stored in the laboratory for a long period and reactivated and/or scaled up using bioreactor for further studies. Even though there are many reports available on marine microbes for the metabolite production, reports on sponge endosymbionts particularly from Lakshadweep are very scanty. Based on the antibacterial activity this study concluded that phylogenetic analysis of endosymbiotic bacteria *Enterobacter* sp from sponge *D. granulosa* of Lakshadweep islands showed significant antibacterial activity against clinical pathogens. Thus, the present study is a preliminary work in the Lakshadweep endosymbiotic bacteria of *Enterobacter* sp TTAG and further studies are in progress in characterization and purification of the secondary metabolites of the host sponge *D. granulosa*.

Conflict of interest statement

We declare that we have no conflict of interest.

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