International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Thematic Special Issue 2022

Original Article

PHYLOGENY OF ENDEMIC MARINE SPONGE *HYATTELLA INTESTINALIS* AND CHARACTERIZATION OF ASSOCIATED BACTERIA

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Received: 16 Apr 2021, Revised and Accepted: 29 May 2022

ABSTRACT

Objective: The main objective of this study was to assess the diversity of culturable bacteria associated with marine water sponges.

Methods: The marine sponge of *Hyattella intestinalis* was collected from Sethubavachatram, a village panchayat in the Thanjavur district of Tamil Nadu state, India. One gram of the sponge sample was cut into small pieces and serially diluted. spread on Zobell 2216E marine agar medium and incubated at room temperate (RT, 24±2oC) for 48 h. Based on morphological features, colonies were randomly picked and purified using streak plates. The linked bacteria strains were given names based on the initials of the sponge from which they were recovered.

Results: The HIB1, HIB2, and HIB3 were isolated from *Hyattella intestinalis*. Among the three, HIB1 colonies were found dominant, and further, the biochemical characterization and 16S rRNA sequence was studied. It revealed that the marine sponge *Lysinibacillus sp.* associated bacteria with HIB1 code.

Conclusion: The HIB1 was closely related to *Lysinibacillus macroides* with 97.81% homology. The NCBI accession number was MZ695813.

Keywords: Sponge, *Hyattella intestinalis*, Sethubavachatram, *Lysinibacillus macroides*, 16S rRNA sequence

DOI: https://dx.doi.org/10.22159/ijap.2022.v14ti.33 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Sponges are primitive metazoans that belong to the Porifera order and are an important part of the benthic environment in marine ecology [1, 2]. Sponges are members of the Spongiidae family and range in size and shape from low encrusting to upright massive. Marine sponges are home to a large variety of microorganisms and have close relationships with a wide range of bacteria. They're abundant and widely distributed in most marine waters, and they've long been thought to be a source of bioactive substances with medicinal promise [2, 3]. Microorganisms found in the mesophyll matrix of sponges may account for 35–60% of the total sponge mass [4-6].

Sponge microbiotas are extremely diverse, with members of seven different bacterial phyla found in sponges. These sponge-associated bacterial symbionts are considered to play a range of roles in the sponge's biology, including removing toxic metabolic wastes, producing bioactive chemicals, and increasing structural rigidity, all of which could be helpful to the host [7, 8]. Sponge microbial diversity has recently been revealed to include at least 26 phyla, and several investigations involving sponge-associated bacteria have been conducted [9, 10]. As a result, the goal of this research was to identify bacteria from the marine sponge *Hyattella intestinalis*.

MATERIALS AND METHODS

Collection and identification of *Hyattella intestinalis*

Sethubavachatram village (fig. 1), a village panchayat in Thanjavur district, Tamil Nadu state, India, provided the marine sponge of H. intestinalis [11, 12]. Sethubavachatram's geo-coordinates are 10.2516118 latitudes and 79.2775316 longitude. Sethubavachatram village's state capital is Chennai. Sethubavachatram is approximately 334.8 kilometres away. All of the acquired samples were cleaned three times with tap water and twice with distilled water to remove any associated clinging animals.

Isolation and purification of bacteria

One gramme of sponge was cut into little pieces and diluted serially. Incubated for 48 h at room temperature (RT, 24 2oC) on Zobell 2216E marine agar medium. Colonies were chosen at random and purified using streak plates based on morphological characteristics. The bacterial strains connected with it were given the initials of the sponge from which they were isolated. Selecting colonies with specific morphological features and moving them to freshly produced media until pure cultures were obtained [13, 14] was how the bacterial isolates were purified.

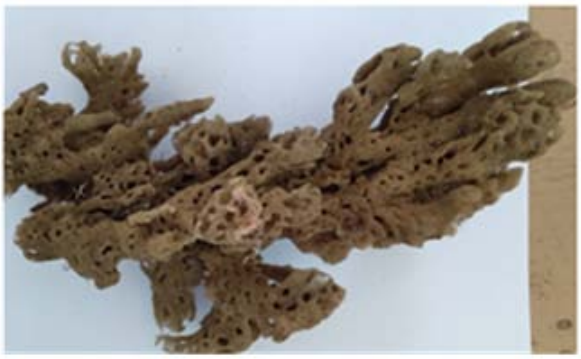

Fig. 1: ***Hyattella intestinalis***

Mass cultivation of total heterotrophic bacteria

After that, a loopful inoculum of bacterial isolates was inoculated into a nutritious broth (pH 7.2) produced with 50 percent old saltwater and kept at 282 °C for 24 h with constant shaking. The broth culture was then transferred to 1000 ml of nutritious broth containing 50% old saltwater and cultivated for 4-5 d with continual shaking [15]. The bacteria were visually spotted in the culture plate [16, 17].

DNA isolation by cetyl trimethyl ammonium bromide (CTAB) method

Bacterial culture

500 l CTAB Extraction Buffer was added to the pellet, stirred thoroughly, and vortexed. 30 min in a 60 °C bath with the

homogenate Following the incubation period, the homogenate was centrifuged at 14,000 x g for 5 min. An equal volume of chloroform/isoamyl alcohol was added (24:1). To separate the phases, vortexed the sample for 5 seconds before centrifuging it for 5 min at 14,000 x g. The aqueous part of the upper phase was transferred to a fresh tube. 0.7 volume cold isopropanol was used to precipitate the DNA, which was then incubated at-20 °C for 30 min. For 10 min, the sample was centrifuged at 14,000 x g. Decant the supernatant without disturbing the particle, then wash with 500 liters of ice-cold 70% ethanol. The ethanol was decanted. Using a SpeedVac, I was able to remove any remaining ethanol. The pellet was dried long enough to eliminate the alcohol, but not long enough to totally dry the DNA. DNA dissolved in 30 litres of TE buffer. It's possible that the pellet will need to be warmed before it dissolves. 1 l RNase solution A was added and incubated for 30 min at 37 °C. The acquired DNA must be quantified [18, 19].

Molecular identification of bacteria isolated from the marine sponge by 16s rRNA sequencing

Column purification

200 l of binding buffer was added to the DNA and thoroughly mixed. Transferred to the column (750 l each time), and spun for 1 minute at 12000 rpm. I added 750 l of wash buffer and spun for 1 minute at 12000 rpm. The wash buffer phase was repeated. Dry spin for 2 min, then add 20 l of elution buffer and spin for 1 minute at 12000 rpm. The resulting DNA must be quantified [20, 21].

PCR conditions

Initial denaturation at 95 °C for 2 min, followed by 30 seconds of final denaturation at 95 °C. 30 seconds at 50 °C annealing 1 minute of 72 °C elongation Rep steps 2, 3, and 4 for a total of 30 cycles. Final Elongation: 72 °C for 10 min, then 4 °C indefinitely. Table 1-lists the primers and sequences that were employed in this study [12].

Table 1: Details of primers

Primer	Sequence	Annealing temperature
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	53 °C
1492R	5'-GGTTACCTTGTTACGACTT-3'	57 °C
ITS-1	5'-TCCGTAGGTGAACATGCGG-3'	53 °C
ITS-4	5'-TCCTCCGCTTATTGATATGC-3'	57 °C

Preparation of PCR reaction mixture

Each PCR reaction contained 1l DNA template (25 ng), 2l 10X reaction buffer, 0.5l MgCl2 (50pM), 1l dNTPs mix (10 mmol), 1l forward primer (10pM), 1l reverse primer (10pM), 0.5l Taq polymerase (5 U/pi), and the final volume 25l will be adjusted with molecular grade water. Standard primers for amplification of the 16srRNA gene are available. In comparison to a 1KB ladder, the 16srRNA gene (1200bp) was amplified from bacterial DNA.

Components

Forward primer: 10pM, Reverse primer: 10pM, 10Mm dNTPs: 10µM, 10X PCR Buffer: 2x, 25Mm Mgcl2:2.5 mm, Template DNA: 25-40ng, Taq polymerase: 5U, Nuclease free water: Make upto 25µl

Gel purification protocol

On the gel, cut the necessary DNA band. Heat at 55 °C for 600 L of Gel Solubilization Buffer until the gel dissolves completely. 200 litres of isopropanol were added, stirred, and put to a column. For 1 minute, spin at 12000 rpm. Added 700 L of wash buffer and spun for 1 minute at 12000 rpm. 2 min of dry spinning Add 20 litres of Elution Buffer and spin for 1 minute at 12000 rpm [22, 23].

Sanger sequencing PCR

The initial denaturation was placed at 95 °C for 2 min, followed by 30 seconds of final denaturation at 95 °C. 30 seconds at 50 °C annealing Finish at 60 °C for 4 min. Rep steps 2, 3, and 4 for a total of 30 cycles. Keep the temperature at 4 °C indefinitely.

Post sequencing and PCR purification

To each well, add 125 Mm 2.5 L EDTA and spin briefly. Using a multichannel pipette, add 35L of Ethanol. Vortexed for 10 min at 2000 rpm, then centrifuged for 30 min at 3510 rpm. Decant Ethanol at 300 rpm using a tissue bed (for 30 seconds, invert the plate). Add 40L of 80 percent ethanol to the wells and centrifuge for 12 min at 3510 rpm. I did the same inverted spin as before. Cover the plate with lint-free tissue and air dry for 30-45 min. Add 13L of HiDi Formamide and spin for a few minutes. Denature for 5 min at 95 °C. In the sequencer [24], place the plate.

Data analysis

The sequencing files will be in AB1 format, which may be viewed with programmes like Finch TV, BioEdit, Chromas Lite, Seq Scanner, and others. Electropherogram peaks can be used to assess the quality of the sequence acquired. Analyze the sequencing data with the use of BLAST server(s) for selected databases.

DNA sequencing and phylogenetic analysis

A Wizard PCR Preps DNA Purification System is used to extract 16S rDNA amplified products from an agarose gel (Promega). A DNA sequencer is used to carry out the sequencing (3130xl genetic analyzer). The retrieved sequences can be compared to bacterial rDNA sequences in the GeneBank, and NCBI BLAST tools can be used to find commonalities. The software Clustal Omega may be used to create phylogenetic trees.

RESULTS AND DISCUSSION

Isolation of *Hyattella intestinalis* associated bacteria

Table 2 shows the number of bacterial isolates connected with the sea sponge H. intestinalis. Only three different physical traits of cultivated bacteria were linked to *Hyattella intestinalis* (table 3). HIB1 (*Hyattella intestinalis* Bacteria 1), HIB2 (*Hyattella intestinalis* Bacteria 2), and HIB325 (*Hyattella intestinalis* Bacteria 325).

Fig. 2 shows the results of the current investigation, which found three distinct morphological colonies, the most prevalent of which were the HIB1 colonies, followed by the HIB2 and the HIB3 colonies.

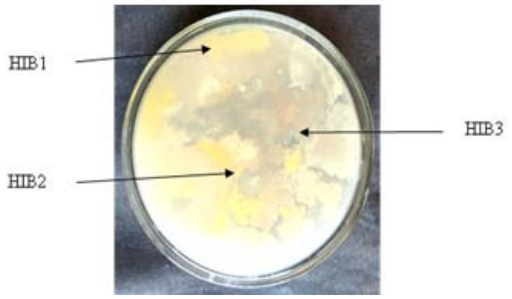

Fig. 2: *Hyattella intestinalis* associated bacteria (10^{-6})

Table 2: Counts of associated bacteria colony from marine sponges *Hyattella intestinalis*

Dilution	CFU/gm
10^{-5}	TNTC
10^{-6}	2.00×10^{8}
10^{-7}	1.10×10^{9}
Control	Nil

Table 3: Morphological characteristics of *Hyattella intestinalis*-associated bacteria (10^{-6})

Isolates	Colony colour	Colony size	Colony form
HIB1	Yellow	Large	Irregular
HIB2	White	Medium	Irregular
HIB3	Light pink	Small	Irregular

In the present study, three different morphological colonies were identified, where the HIB1 colonies were dominant, along with HIB2 and HIB3 colonies (fig. 2).

Fig. 3: Gram staining of HIB1 Isolates

Morphological and biochemical characterization of HIB1 bacteria isolates

The morphology of microorganisms that have been isolated It was determined that HIB1 belonged to the gram-negative bacteria group; the Indole test was negative; the Citrate Utilization Test, Methyl red test, and Voges-Proskauer tests were all positive; and all controls were negative (table 4, fig. 3 and 4).

Table 4: The biochemical characterization of HIB1 bacteria isolates

Biochemical characteristics	Result
Gram stain	-
Indole	-
Citrate Utilization	+
Methyl red	+
Voges-Proskauer	+

(+) = Positive; (-) = Negative

Amplification of partial 16S ribosomal RNA of HIB1 bacteria isolates

The morphology of microorganisms that have been isolated It was determined that HIB1 belonged to the gram-negative bacteria group; the Indole test was negative; the Citrate Utilization Test, Methyl red test, and Voges-Proskauer tests were all positive; and all controls were negative (table 4 and fig. 3 and fig. 4).

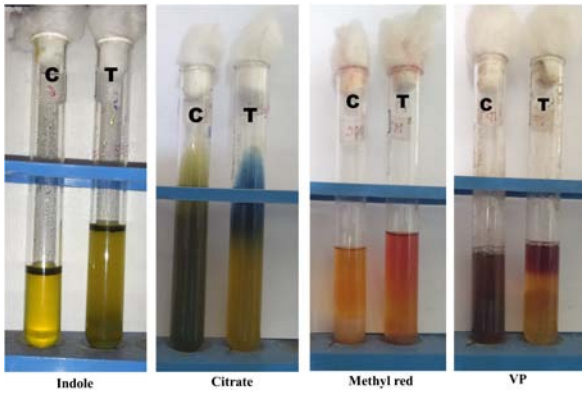

Fig. 4: Biochemical tests of HIB1 Isolates

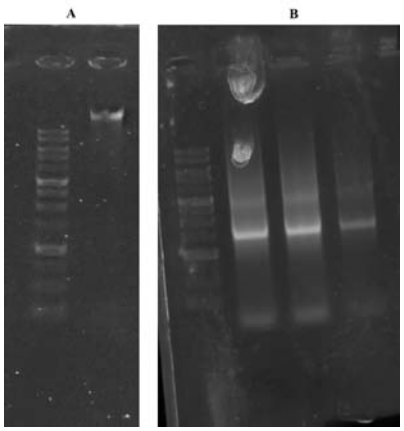

Fig. 5: (a) Genomic DNA isolated from bacteria compared with 1KB ladder (b) 16srRNA gene (1200bp) amplified from bacterial DNA as compared with 1KB ladder

The 16srRNA gene (1200bp) amplified from bacterial DNA and compared to a 1KB ladder are shown in fig. 5(a) and fig. 5(b).

DNA sequencing and Phylogenetic contractions of HIB1 bacteria isolates

Based on DNA sequencing, the DNA amplification of HIB1 isolates represented around 1200bp and shown in fig.6.

Bacterial sequence

>A BAC_16S_G11. ab1

AATACCGAATAATCTCTGCTTCATGGAAAAGACTGAAAGACGGTTTCG
GCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTA
ACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCAGTGA
AGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTAC
AGTAGTAACTGGCTGTACCGACGGTACCTTATTAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTG
AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTG

AGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTA
GAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAAC
TGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCG
CCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCCCGTTGACCACTGTAGAGATATAGTTTCCCCTTCGGGGGCA
ACGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCAT
TTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAA
TCCGATAAAGTCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACA
TGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATA
CGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAA
CACCCGAAGTCGGTGAGGTAACCT

Hit: *Lysinibacillus analicula* strain LNHL43 16S ribosomal RNA gene

Percent identity: 97.81%, E value: 0.0, Query coverage: 81%

Accession no: MZ695813 (Plate 1) Version: MZ695813.1

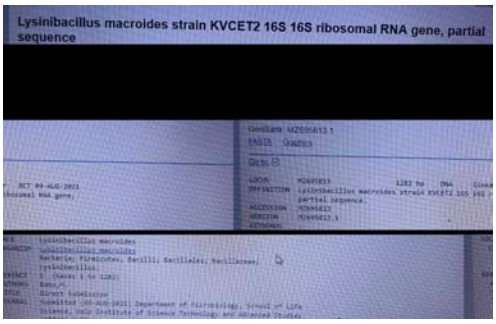

Fig. 6: The DNA sequencing results of HIB1 isolates (screenshot of NCBI)

Based on the results of the BLAST homology analysis, the HIB1 isolates were found to be closely related to *Lysinibacillus analicula* strain LNHL43 in terms of 16S ribosomal RNA gene sequence homology, with 97.81 percent similarity to the LNHL43 strain. Fig. 7 depicts phylogenetic trees of HIB1 isolates based on DNA sequences.

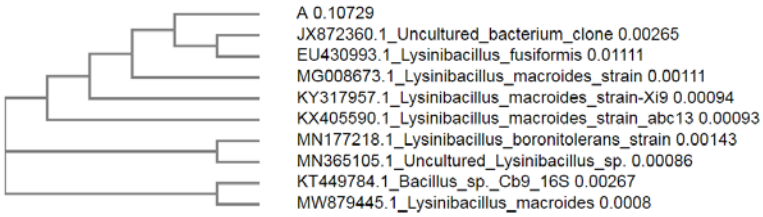

Fig. 7: The phylogenetic trees of HIB1 isolates

CONCLUSION

The current analysis came to the conclusion that three different colonies (HIB1, HIB2, and HIB3) had been found. The biochemical characterisation and 16S rRNA sequencing of the HIB1 colonies were investigated further in order to determine which colonies were dominant among the three. The marine sponge *Lysinibacillus sp*. was discovered to be the causative agent. Bacteria that are associated with the HIB1 code. With 97.81 percent homology to *Lysinibacillus analicula*, the HIB1 was found to be closely related to this bacterium. As a result, the bacteria associated with the sponge *Lysinibacillus analicula* are thought to be the most likely source of bioactive chemicals for isolation purposes.

FUNDING

Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

We declare there is no conflict of interest.

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