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Original Article

Preliminary Investigation on the Nature of Bacteria Associated with the Giant Barrel Sponge, *Xestospongia testudinaria*, Using Culture-Dependent Methods

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Abstract. Within the Singapore marine environment, the diversity of bacterial communities in sponges is largely underexplored. This study is a preliminary investigation, using culture-dependent methods, on the bacterial community associated with the giant barrel sponge, *Xestospongia testudinaria*, from three locations, namely Pulau Hantu, P. Kusu and P. Semakau. Of the 84 marine microbial colonies isolated in this study, the phylogenetic analysis of 20 bacterial strains was successfully carried out. These bacterial isolates belonged to either phylum Proteobacteria or Firmicutes. Marine bacteria of the genera *Bacillus* and *Vibrio* were found in sponge samples from all three locations, while other bacteria genera, such as *Labrenzia* and *Pseudoalteromonas*, were only successfully isolated from samples from P. Hantu and *Halomonas*, *Marinobacter*, *Pseudomonas* and *Phaeobacter* detected from P. Semakau derived samples. In addition, two marine bacterial strains, S1B11 and S2A10, showed sequence identity of below 80% with their nearest relative of *Labrenzia alba* and *Bacillus vietnamensis*, respectively and could represent novel bacterial species. Majority of the isolated bacterial strains was found clustered in high sequence identity with bacterial species known to produce bioactive specialized metabolites, suggesting that these associated microbes could provide chemical defense for the host sponge and contribute to its resilience in Singapore waters.

Keywords: *Xestospongia testudinaria*; marine bacterial symbionts; phylum Proteobacteria, phylum Firmicutes, culture-dependent method

Introduction

The occurrence of the giant barrel sponges, *Xestospongia* sp., is ubiquitous in tropical reefs in Indo-Pacific and Atlantic (McGrath et al., 2018). These sponges are long-lived, able to grow beyond a meter and capable of processing large volumes of seawater as well as participating in nutrient cycling pathways (Fiore et al., 2015). Some species can measure up to a base diameter of greater than 2.5 m, with coverage as high as 9% in some reef systems. Currently, more than 30 *Xestospongia* species, including *X. testudinaria* (Lamarck, 1815), *X. bergquistia* (Fromont, 1991), *X. muta* (Schmidt, 1870) and *X. exigua* (Kirkpatrick, 1900), have been described. Within the Southeast Asian region, these species are typically found in shallow tropical coral reefs as well as deeper waters. Recent molecular-based research revealed that the giant barrel sponges belong to a pan global species complex with 17 genetic clusters, consisting of up to nine cryptic species (Swierts et al., 2017; Evans et al., 2021). Morphologically similar species can occur sympatrically and could feature differences in their physiological roles. Members of the *Xestospongia* genus have also been well studied for their rich source of secondary metabolites, including steroids, terpenoids and alkaloids, with associated biological activities ranging from anti-inflammatory, antimicrobial to anticancer property (Zhou et al., 2010).

Xestospongia testudinaria is a well-known high microbial abundance sponge and has been studied due to its ecological importance as a habitat engineer and source of therapeutic drug agents (McGrath et al., 2018). This species is also one of the most common sponge species in Singapore waters (Lim et al., 2012). Several studies reported on the microbial community of *X. testudinaria* collected from different locations, including the Red Sea, Malaysia, north and south Sulawesi, Northwest Java, East Kalimantan, West Papua, Vietnam, South China Sea as well as Singapore. Using culture-independent methods, these studies revealed that *X. testudinaria* tends to have similar microbial communities dominated by phyla Acidobacteria, Actinobacteria, Chloroflexi and Proteobacteria (Pires et al., 2019). Several functional roles have been attributed to the sponge associated microbes which could contribute to the resilience of the host animal to environmental perturbations. For instance, microbial genes involved in the nitrogen cycle, including nosZ, nifD, nirK, norB and nrfA genes, were found to be enriched in *X. testudinaria* sponge samples (de Voogd et al., 2015). A study by Cleary and co-workers found *X. testudinaria* to be enriched for KEGG orthologs related to the toxins-antitoxin system, nutrient starvation and export of heavy metals (Cleary et al., 2015). Moreover, studies involving the isolation of associated microbes from the host samples revealed their microbial extracts exhibited potent antibacterial and cytotoxic activities, suggesting that these microbial symbionts provided sponge chemical defense (Cita et al., 2017).

In view of the ecological importance of *X. testudinaria* as well as its common occurrence in Singapore waters, a pilot scale study was initiated to investigate the nature of bacteria associated with samples of *X. testudinaria* collected from three locations, namely Pulau Hantu, P. Kusu and P. Semakau, Singapore, using culture dependent methods. Previous microbiome studies conducted on local *X. testudinaria* used culture-independent approaches, such as 16S rRNA gene barcoded pyrosequencing, to access the diversity of associated microbes (Deignan et al., 2023). In contrast, this study is based on the integration of culture-dependent method, using three different marine media, as well as phylogenetic analysis to uncover associated bacteria from *X. testudinaria* at three reef systems in Singapore. The culture-dependent approach can complement previous culture-independent studies and has implications for further studies, such as uncovering the biotechnological/therapeutic potential and ecological roles of isolated marine bacterial strains.

Materials and Methods

Sponge Samples

Samples of *X. testudinaria* were collected by SCUBA, at depth of about 4.5 m, from three different locations at the southern islands in Singapore, including Pulau Hantu (N 1.2261°61, E 103.7500°), P. Kusu (N 1.2228°, E 103.8611°) and P. Semakau (N 1.2061°, E 103.7619°) (Figure 1). Sponge samples were carefully removed with a dive knife and placed in separate Ziploc® bags containing seawater. At each collection site, small amounts of sponge tissues were removed from three different colonies, making a total of nine different sponge specimens collected. Once brought to the surface, sponge specimens were placed in cooler box and transported to laboratory at NIE within 2 h for further processing.

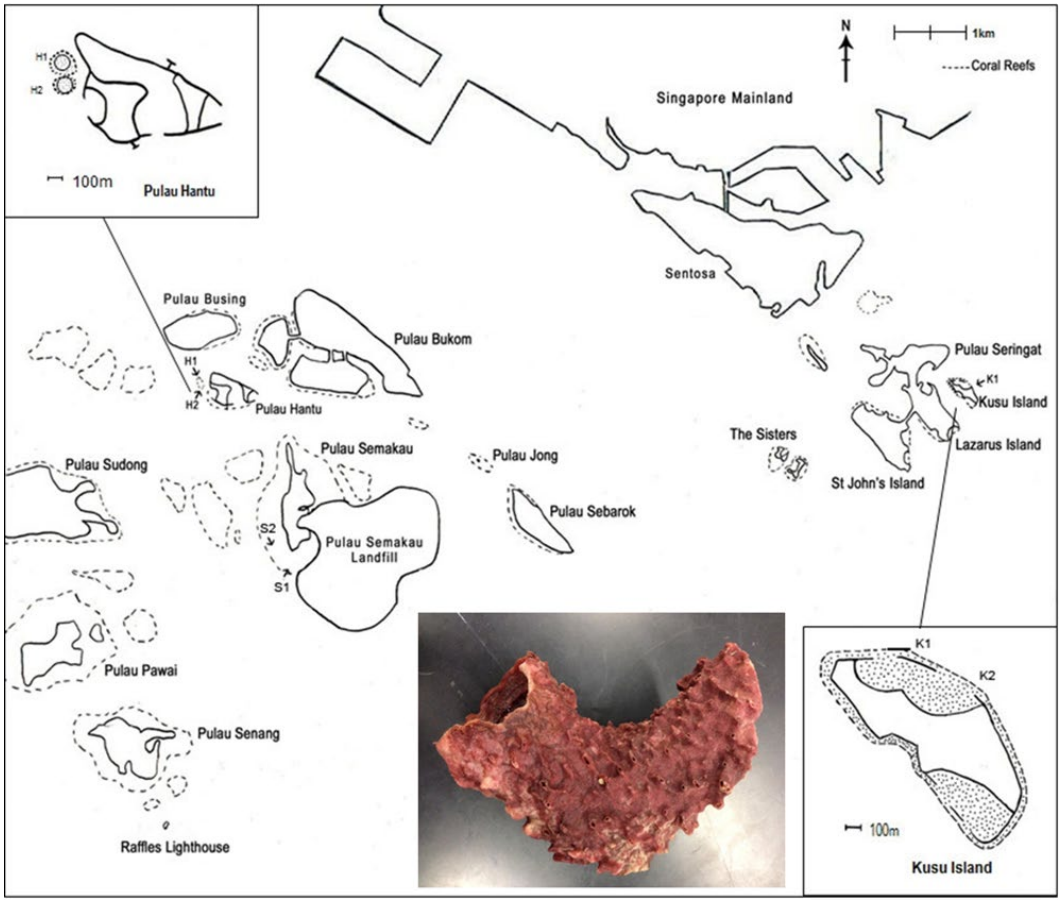

Figure 1. Map of southern reef systems in Singapore indicating collection sites of *X. testudinaria* (picture inserted) at P. Kusu (K1 and K2), P. Hantu (H1 and H2) and P. Semakau (S1 and S2). (Photograph by: Yu Mun Chan)

Marine Media

All marine agar plates used for the isolation of marine bacteria from *X. testudinaria* samples were prepared 24 h before each field collection. Three different types of marine media, namely media M1, M2 and M3, were used for the pilot scale isolation of the sponge associated marine bacteria. These media have been used by researchers for the purpose of enumeration of heterotrophic bacteria and/or for the isolation of actinomycetes (Grein et al., 1958). Medium M1, 1 L of agar contained 2.0 g of mannitol, 0.1 g of L-asparagine, 2.0 g of calcium carbonate, 0.5 g of dipotassium phosphate, 0.1 g of magnesium sulfate, 0.01 g of iron (II) sulfate, 0.001 g of vitamin B1, 0.001 g of vitamin B6, 0.001 g of vitamin lactoflavin,

0.001 g of nicotinic acid, 0.001 g of biotin, 0.001 g of L-phenylalanine, 0.0003 g of L-alanine, and 18 g of agar dissolved in 1 L of artificial seawater (33.3 g of sea salts dissolved in 1 L of distilled water). Medium M2, 1 L of agar contained 10 g of soluble starch, 4 g of yeast extract, 2 g of peptone and 18 g of agar dissolved in 1 L of artificial seawater. Lastly, medium M3, 1 L of agar contained 0.5 g of soluble starch, 0.5 g of yeast extract, 0.25 g of tryptone, 0.75 g of peptone, 0.5 g of glucose, 0.3 g of dipotassium phosphate, 0.024 g of magnesium sulphate, 0.3 g of sodium propionate, and 18 g of agar dissolved in 1 L of artificial seawater. In addition, for every liter of the medium prepared, 0.015 g of nalidixic acid and 0.05 g of potassium dichromate were added to inhibit the growth of fast-growing gram-negative bacteria and growth of fungi. All media were adjusted to pH 7.2 to 7.4, using sodium hydroxide or hydrochloric acid to allow optimal growth of marine bacteria on the agar.

Isolation of Sponge Associated-Marine Microbes

Each sponge sample was thoroughly washed with sterile artificial seawater to remove any surface or loosely attached bacteria. On a sterile cutting surface, the mesohyl region of each sponge specimen was cut into 1 cm^3 sections with a sterile pair of scissors for isolation of associated marine bacteria. Each 1 cm^3 sponge sample was then homogenised in 10 mL of sterile artificial sea water using a sterile mortar and pestle and thoroughly ground for 2 min. This mixture of homogenised sponge tissue in artificial seawater was designated as the 10° dilution sample. As there were nine different sponge samples, a total of nine sponge samples at 10° dilutions were prepared. Each sponge mixture was serially diluted tenfold in sterile artificial seawater to pro-vide 10^{-1}, 10^{-2} and 10^{-3} dilution mixtures. This created a total of 36 different dilution mixtures from the original nine sponge samples that were then plated on the marine agar media M1, M2 and M3.

For each sponge dilution mixture, 100 μL was inoculated onto each agar plate and spread evenly with a plate spreader and allowed to dry in a Class II biosafety cabinet. For every medium and dilution, duplicate spread plates were prepared, e.g., a 10° dilution of one homogenised sponge sample would be plated onto two each of M1, M2 and M3 agar plates. Thus, for every sponge sample collected, a total of 24 inoculated plates were prepared and for the nine homogenized sponge samples, a total of 216 inoculated plates were obtained. All inoculated plates were incubated at 28 °C for a duration of five weeks. On a weekly basis, all inoculated agar plates were examined, and bacterial morphology and growth were characterised based on colour, elevation, form and sizes of the various colonies observed. Distinct colony morphotypes that were observed on the inoculated plates were then selected and isolated by restreaking the bacterial colony onto new agar plate using disposable plastic sterile inoculation loops. Streak plates contained the same media that the colonies were originally identified and selected. Only successfully restreaking plates with individually observable bacterial colonies were selected for subsequent DNA extraction and sequencing of 16S rRNA genes.

Characterization of Marine Bacterial Isolates Using Gram Staining

Marine bacterial species that were successfully isolated were subsequently Gram stained to characterize marine bacterial cells. Using a disposable inoculation loop, single bacterial colonies from the streak plates were transferred and mixed with a drop of distilled water on a clean glass slide conducted in a Class II biosafety cabinet. The mixture of bacterial cells and water was then spread thinly across the glass slide before being placed under a flame to fix the cells onto the slide. This step helped to affix the bacterial cells onto the slide and prevent them from sliding off during the washing steps of Gram staining. A primary stain of crystal violet was added onto the slide for 20 s and rinsed off using distilled water. Next, Gram's iodine solution was added to the slide to act as a mordant and allow a complex to

form between the crystal violet and iodine. After 20 s, iodine was again rinsed off with distilled water. A decolouriser of 95% ethanol was then applied to the sample for approximately 5 s. This step allowed the complex of crystal violet-iodine to be retained in the outer cell wall of gram-positive cells but not gram-negative cells. Lastly, a counterstain of saffranin was added to the slide for 15 s. As the Gram-negative bacterial cells were previously de-colourised, they were stained red, but the purple colouration that was retained in the gram-positive cells was not affected by the saffranin, which was lighter coloured compared to crystal violet. The slides prepared by Gram stainning were then viewed under a light microscope using an oil immersion lens at 1000× magnification, with images captured using an Olympus camera.

Small-Scale Culturing of Selected Marine Bacterial Isolates

Single bacterial colonies from the isolated bacterial streak plates were picked and inoculated in 50 mL of marine liquid medium. The liquid medium used for the liquid media was the same medium in which the colonies were originally isolated and streaked onto. These bacterial isolates were grown in liquid media for 5 – 7 days at 28 °C until cells were at the exponential growth phase.

Extraction and Isolation of Marine Bacterial DNA

Several commercial kits were used to attempt extraction of bacterial DNA; how-ever, DNA could not be extracted successfully as marine bacteria were highly resistant to lysozyme digestion. Thus, a modified protocol involving the use of liquid N_2 to lyse cells was used (Lee et al., 2003). Cells that were grown in liquid media previously for 5 – 7 days were centrifuged at 4000 g for 10 min to pellet the cells. The supernatant was discard-ed while the cell pellet was then transferred to a sterile mortar and pestle and liquid N_2 was then added. The frozen cells were then ground using the pestle until the con-sistency of a fine powder. Ten milliliters of resuspension buffer (50 mM Tris-Cl pH 8.0, 30 mM EDTA pH 8.0, 0.1 M NaCl, 2% SDS, 0.2% polyvinylpyrrolidone, 0.1% β-mercaptoethanol) was then added to the ground cell pellet. Additional liquid N_2 was then added to the mixture, and the mixture was ground until it thawed before adding 2.5 mL of 5 M NaCl and 1.5 mL of 10% cetyltrimethylammonium bromide (CTAB). The high concentration of NaCl together with CTAB helped to remove polysaccharides during the DNA extraction process. Proteinase K was then added to the mixture to a final concentration of 2.0 mg/mL and the mixture was incubated at 50 °C for 1 h. After incubation, the mixture was centrifuged at 4000 g at 4 °C for 10 min to pellet the cell debris. Carbohydrates precipitated by CTAB appeared as a white layer above the liquid phase. Only the liquid phase was collected.

To the liquid phase, an equal volume of 25:24:1 phenol-chloroform-isoamyl alcohol was added and mixed gently until it was homogenous. The mixture was then centrifuged at 5000 g at 4 °C for 10 min. After centrifugation, the mixture separated into two layers. The aqueous layer (top layer) was then removed and transferred to a new falcon tube and an equal amount of chloroform was added to the aqueous phase and mixed gently until homogeneous. The mixture was then centrifuged again at 5000 g at 4 °C for 10 min. Again, after centrifugation, the top aqueous layer was removed and the step involving the addition of chloroform and centrifugation was repeated. The top aqueous layer was removed after centrifugation and transferred to a new falcon tube. RNase A was then added to a final concentration of 100 μg/mL and the mixture was incubated at 37 °C for 30 min. After incubation, DNA was precipitated by adding an equal volume of isopropanol and mixed by inversion. DNA that was precipitated was centrifuged at 5000 g for 10 min to form a pellet, and the supernatant discarded. The DNA pellet was then washed with a small volume of 70% ethanol twice and allowed to dry overnight at room temperature to remove residual ethanol.

The dried DNA was subsequently redissolved in 300 μL of TE buffer and stored at –20 °C until future use.

Phylogenetic Analysis of Selected Marine Bacterial Isolates

The concentration and quality of the bacterial DNA extracted was checked by running the DNA sample on a 1% agarose gel, together with the use of the nanodrop (ND-1000). The nucleic acid programme was selected, and DNA samples which had the absorbance ratio (A260/A280) between 1.8–2.0 and concentration of at least 10 ng/μL were selected for 16S rRNA gene amplification. Approximately 1500 bp of the 16S rRNA gene fragment was amplified by PCR, using the purified extracted genomic DNA from the various bacterial isolated cultures. The primer set used were the uni-versal primers 27F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1500R (5′-AGAAAGGAGGTGATCCAGCC-3′) (Woese et al., 1983). Thermal cycling was done using Thermo Scientific Taq Polymerase and Taq Buffer (with KCl). The initial denaturation was at 95 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 40 s and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 10 min. Thermal cycling was performed with an Applied Biosystems thermocycler.

Thermal cycling reactions were first tested and optimised in 10 μL to determine the amounts of template DNA and MgCl needed to be added for each reaction, before scaling up to 50 μL reaction volumes. For the 10 μL PCR reactions, the amplified 16S rRNA gene DNA bands were confirmed by running a 1% agarose gel and visualised using GelRed under UV light. For the optimised 50 μL PCR reactions, 10 μL of the amplified 16S rRNA gene bands were confirmed and visualised by running a 1% agarose gel amended with GelRed. If the 1500 bp 16S rRNA gene band was observed in the agarose gel, the remaining 40 μL of the PCR product was then purified using the PureLink PCR Purification Kit (Invitrogen, USA) before the concentration and quality of the PCR product checked using the nanodrop (ND-1000) and subsequently out-sourced for gene sequencing, with 27F being used as the sequencing primer. The quality and concentration of the cleaned up amplified 16S rRNA gene PCR products were checked using the nanodrop (ND-1000), and only samples with the absorbance ratio A_{260}/A_{280} of 1.8-2.0, with a minimum concentration of 40 ng/μL were selected to be sent for DNA sequencing by Axil Scientific, Singapore.

DNA sequencing results of amplified 16S rRNA gene fragments were viewed using MEGA 6. Regions in which the DNA sequence had a good signal were selected for each of the samples and were used for analysis of the partial 16S rRNA gene sequences. The gene sequences were then compared to those in the GenBank database using the basic local alignment search tool (BLAST), which helped to identify the closest NCBI (BLASTn) relatives of the bacterial sample strain that was being studied. The 16S rRNA gene sequence was then extracted from GenBank and subsequently used for further phylogenetic analysis. Alignment of partial 16S rRNA gene sequences of the marine bacterial samples and the 16S rRNA gene sequences of their closest relatives was carried out using MEGA 6 and ClustalW for multiple alignments of the various DNA sequences selected. Alignment of sequences was carried out separately for samples for P. Hantu, P. Kusu and P. Semakau and their nearest relatives; an additional alignment was carried out for the combined 16S rRNA gene fragments of all three locations and their nearest relatives. Additional phylogenetic analysis was carried out via the neighbour joining method to show the phylogenetic relationships of bacterial sample strains found in *X. testudinaria* and their nearest relatives. The phylogenetic trees generated were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were

computed using the p-distance method and are in the units of the number of base differences per site.

Results

Isolation and Characterization of Marine Microbes from Samples of X. testudinaria

Three different types of seawater media, including M1, M2 and M3, were used to isolate and culture marine bacteria associated with *X. testudinaria*. The weekly growth of new bacterial colonies that appeared on each of these agar media plates containing sponge extracts at different concentrations was recorded over a period of 5 weeks. Medium M2 was the most general and the least selective of the three marine media, thus large amounts of microbial growth were observed during the first week on M2 compared to M1 and M3 media. The growth of bacteria was also rapid where many of the M2 media plates were covered in different coloured and thick growths of bacterial lawn compared to the small number of individual bacterial colonies observed on the M1 and M3 plates during the first week of bacterial characterisation. During weeks 3 to 5 of bacterial growth characterisation, new bacterial colonies could still be observed growing on media M1 and M3, as the plates had space available for growth of the slower growing bacterial colonies. However, as the plates containing media M2 were generally covered completely in the first two weeks, it was generally rarer to observe new bacterial growth in these media plates during the later weeks of characterisation.

Marine bacterial colonies successfully isolated and restreaked onto their appropriate agar media during the five weeks of characterisation were Gram-stained to allow bacterial cells to be visualized more easily using a light microscope. Figure 2 shows a range of selected bacterial strains isolated from samples of *X. testudinaria* obtained from three different sites. The isolated marine bacterial strains had colonies that were pigmented, ranging from dark red, orange, pink, purplish-black and black (Figure 2). White or colourless bacterial colonies were also observed on the inoculation plates (Figure 2). We noticed that a majority of the isolated bacterial strains produced large amounts of extracellular materials, which made genomic DNA extraction challenging. However, there were a small number of bacterial strains isolated from each of the sponge samples that did not contain any extracellular materials. From bacterial strains isolated from P. Hantu, only seven bacterial strains, including S1B10, S1A13, S2A10, S2B15, S3B5, S3A13 and S3A26 did not produce large amounts of extracellular materials. A similar number of bacterial strains were also observed from bacterial strains isolated from P. Kusu. Only seven bacterial strains, including K1B3, K1B8, K1B9, K1B10, K2B8, K3A13 and K3B16, did not produce large amounts of extracellular materials. Lastly, from bacterial strains isolated from P. Semakau, only sponge samples 1 and 3 had bacterial isolates that did not produce extracellular materials (e.g., P1A4, P1B5, P1B6, P1B7, P1A17, P1B19 and P1B25).

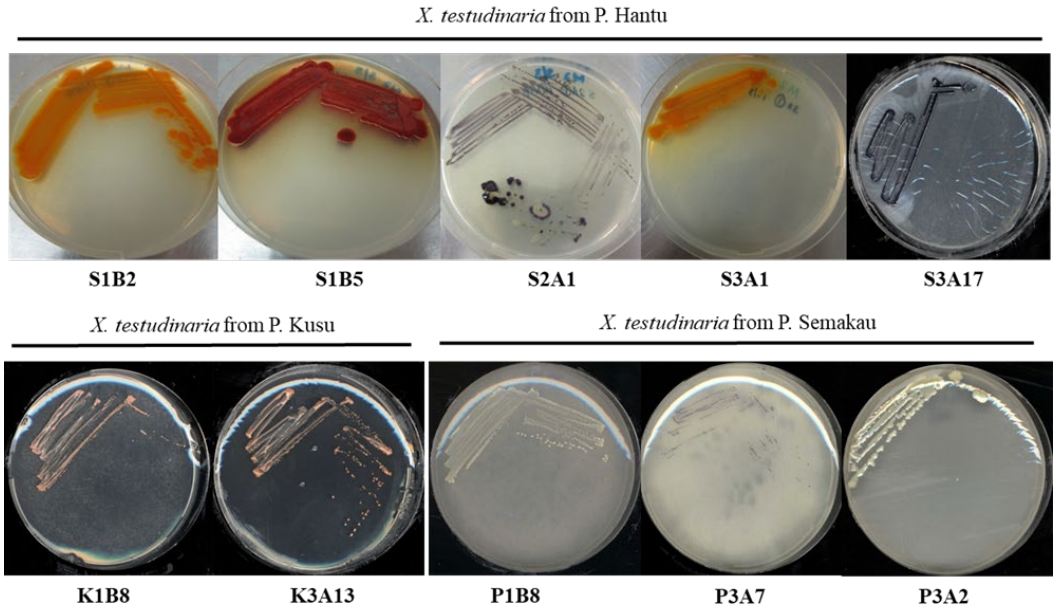

Figure 2. A selection of ten isolated marine bacterial colonies plated on marine agar petri dishes (100 mm × 15 mm) from samples of *X. testudinaria* obtained from P. Hantu, P. Kusu and P. Semakau.

Extraction and PCR of Genomic Marine Bacterial DNA Material

Out of the 84 marine bacterial strains isolated, we successfully extracted genomic DNA from 40 strains, based on the modified protocol using liquid N_2 (Lee et al., 2003), making the success rate of the DNA extraction protocol about 48% (Table 1). Failure to extract genomic DNA was observed when DNA failed to precipitate out at the end of the extraction process. The initial methods of using commercial plant genomic or bacteria genomic DNA extraction kit yielded only about a 10% success rate, and the modified protocol using liquid N2 for DNA extraction resulted in the highest rate of success.

With the bacterial genomic DNA extracted, the 16S rRNA gene fragments were then PCR amplified using the universal primers 27F and 1500R (Woese et al., 1983). PCR of the 16S rRNA gene fragment resulted in a band that is approximately 1500 bp in length. Out of the 40 bacterial genomic DNA samples that underwent PCR, 29 of the samples showed bands that were approximately 1500 bp, which most likely presented the amplified 16S rRNA gene. Based on these results, the success rate of the PCR protocol is determined to be 73% (Table 1). Although a band of approximately 1500 bp in size was present on the DNA gel electrophoresis, the process of PCR clean-up and subsequent nanodrop showed that a number of samples had concentrations that were below 10 ng/μL and had an absorbance ratio (A_{260}/A_{280}) that was not within 1.8–2.0.

Table 1. Number of isolated marine bacterial colonies from *X. testudinaria* collected from various locations and their success rates for the genomic DNA extraction and PCR of the 16S rRNA gene fragment.

Location	# Isolated Bacterial Colony	# Successful DNA Extraction	Success Rate of DNA Extraction (%)	# Samples with 16S rRNA Band Detected	Success Rate of PCR (%)
P. Hantu	31	16	52	12	75
P. Kusu	25	14	56	8	57
P. Semakau	28	10	36	9	90
Total	**84**	**40**	**48**	**29**	**73**

Phylogenetic Analysis of Selected Marine Bacterial Isolates

Among the samples that were sent for gene sequencing, several samples when viewed using the programme MEGA 6 showed bad sequence signals, and subsequently could not be identified based on the sequence data. Thus, among the 29 amplified PCR products, only 20 bacterial strains had DNA sequences that were of high enough quality to be aligned using BLAST with known 16S rRNA gene sequences in the GenBank database (Table 2). Bacterial PCR samples that could be aligned using BLAST were derived from eight, three and nine marine bacterial strains obtained from sponge samples at P. Hantu, P. Kusu and P. Semakau, respectively (Table 2). This resulted in a success rate of the protocol at 69%. Moreover, among the isolated bacterial strains that were successfully sequenced, most of these strains were isolated from medium M1, with 55% of the sequenced bacterial strains from media M1, 15% from M2 and 30% from M3.

Table 2. Marine bacteria isolated from samples of *X. testudinaria* collected at three locations in Singapore.

Location	Code	Medium	Week of Growth	Nearest Type Strain	Sequence Identity (%)
Pulau Hantu	S1A9	M1	1	*Bacillus altitudinis* (KM369867.1)	100
	S1B10	M3	1	*Bacillus altitudinis* (KM369867.1)	99
	S1B11	M1	2	*Labrenzia alba* (FR750958.1)	63
	S1A13	M3	3	*Staphylococcus warneri* (KM660697.1)	100
	S1B16	M1	4	*Bacillus altitudinis* (KM369867.1)	100
	S1A17	M3	4	*Vibrio tubiashii* (CP009354.1)	99
	S2A10	M3	1	*Bacillus vietnamensis* (KF933662.1)	79
	S3A22	M1	4	*Pseudoalteromonas lipolytica* (KJ917337.1)	100
Pulau Kusu	K3A3	M1	1	*Vibrio neptunius* (EU834014.1)	99
	K3A13	M3	3	*Bacillus subtilis* (KF009681.1)	97
	K3A14	M1	3	*Vibrio alginolyticus* (JN188409.1)	100

Pulau Semakau	P1B8	M1	2	*Phaeobacter daeponensis* (NR_044026.1)	99
	P1B15	M3	2	*Marinobacter lacisalsi* (NR_044392.1)	98
	P2A1	M1	1	*Vibrio sinaloensis* (JN871696.1)	99
	P2A3	M2	1	*Vibrio fortis* (KJ577028.1)	100
	P2A6	M1	4	*Vibrio fortis* (KJ577028.1)	99
	P3A2	M2	2	*Bacillus altitudinis* (KM253188.1)	100
	P3A9	M1	2	*Pseudomonas pachastrellae* (AY880300.1)	100
	P3B12	M2	2	*Bacillus altitudinis* (KM369867.1)	99
	P3B16	M1	5	*Halomonas cupida* (AB681327.1)	99

The BLAST analysis carried out via search through GenBank revealed that the 20 bacterial isolates belonged to nine genera: *Labrenzia*, *Halomonas*, *Pseudomonas*, *Marinobacter*, *Phaeobacter*, *Pseudoalteromonas*, *Staphylococcus*, *Bacillus* and *Vibrio*. Most of the marine bacterial strains were from the genera *Bacillus* and *Vibrio*, comprising of 35% and 30% of all isolated strains respectively. Among the 20 bacterial strains, 60% of the strains isolated were phylum Proteobacteria, while the remaining 40% were phylum Firmicutes. Phylogenetic analysis was carried out on the 20 bacterial isolates by comparing the 16S rRNA gene sequences of the isolated strains and the 16S rRNA sequences of their nearest relatives in the NCBI GenBank database. Neighbour joining phylogenetic trees were then constructed for the isolated bacteria strains with their nearest relatives (Figure 3). In general, both *Vibrio* and *Bacillus* species were found as-sociated with sponge samples from all three locations (Table 2). In addition, *Pseudoalteromonas*, *Staphylococcus* and *Labrenzia* species were only identified in bacterial sample associated with sponge samples from P. Hantu, while *Pseudomonas*, *Halomonas*, *Marinobacter* and *Phaebacter* species were found in bacterial strains obtained from sponge samples derived from P. Semakau.

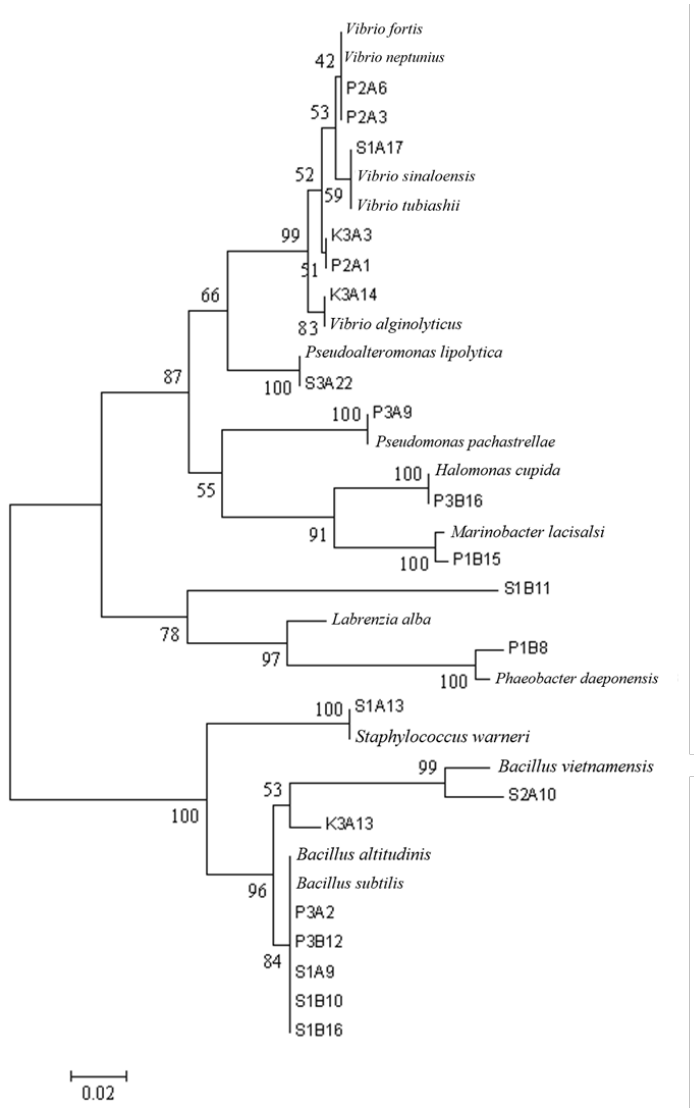

Figure 3. Neighbour joining phylogenetic representation of culturable bacteria isolated from *X. testudinaria* from all three locations.

Discussion

This preliminary study investigates the nature of marine bacteria associated with the ecologically important giant barrel sponge, *X. testudinaria*, collected from three reef systems in Singapore using culture-dependent method. Three marine media, including M1, M2 and M3, were used for the isolation of marine bacteria. These marine media and others have been used successfully by researchers for the isolation of diverse marine microbes from different marine samples, such as sediments and macroorganisms. In fact, many of these studies involved the use of these marine media to isolate and culture marine bacteria for production of novel bioactive metabolites as well as comparative phylogenetic analysis (Zhang et al., 2008).

Different microbial growth patterns were observed using these marine media. An explanation for the differences in the growth rates and quantity of bacteria found on the different media plates is due to the different amounts of nutrients present in the media. For instance, M2 plates contained 10 g/L of soluble starch as a carbon source, which is much higher in concentration as compared to M1 and M3 media. The higher concentration of carbon source in medium M2 could likely facilitate the growth of fast-growing bacteria

which tended to cover the plate over a short period of 2 to 3 weeks. The smaller amounts of carbon source in media M1 and M3 most likely explained the smaller number of bacterial colonies and slower growth rates observed. Regardless on the differences in media compositions, the recovery of associated marine bacteria from sponge samples using media M1 to M3 in this study is consistent with reports by other researchers for the isolation of heterotrophic microbes from environmental marine samples (Jensen et al., 1991).

Of the 84 marine bacterial strains isolated, slightly less than 50% had their genomic DNA successfully extracted based on the DNA extraction protocol by Lee et al. (2003) as compared to only 10% success rate using commercial DNA extraction kits. The low bacterial DNA yield from using these commercial kits could be due to the large amounts of extracellular material that were secreted by many bacterial strains, which tended to clog up the columns of the DNA extraction kit, preventing the binding and subsequent elution of DNA material. In addition, the use of lysozymes to break down bacterial cell walls to release the cell contents was not successful, as the cell walls of these bacteria appeared to be resistant to lysozymes. Thus, for the contents of the cells to be released, liquid N_2 and SDS were used together to lyse the cells mechanically and chemically. As this process required the physical grinding of the frozen cell pellet using a mortar and pestle, it was one of the rate-limiting steps in the genomic DNA ex-traction process. The addition of β-mercaptoethanol, a strong reducing agent, pre-vented the oxidation of phenolic compounds in the cell contents, while addition of polyvinylpyrrolidone removed phenolic compounds by forming hydrogen bonds with them. Moreover, the addition of NaCl and CTAB facilitated the precipitation and removal of polysaccharides during the extraction process, while allowing the nucleic acids to stay in solution.

From phylogenetic analysis, the majority of the 20 isolated marine bacterial strains had high percentage identity (more than 97%) with known bacterial species, including *Bacillus altitudinis*, *B. subtilis*, *Staphylococcus warneri*, *Vibrio alginolyticus*, *V. tubiashii*, *V. neptunius*, *V. sinaloensis*, *V. fortis*, *Pseudoalteromonas lipolytica*, *Phaeobacter daeponensis*, *Marinobacter lacisalsi*, *Pseudomonas pachastrellae* and *Halomonas cupida* (Figure 3 and Table 2). Two marine bacterial strains, S1B11 and S2A10 isolated from sponge samples from P. Hantu, could potentially be novel strains due to their low percentage identity with their nearest bacterial match with *Labrenzia alba* at 63% and *Bacillus vietnamensis* at 79%, respectively. A percentage identity of below 97% is often considered as possible new microbial species. Several of the marine bacterial strains with high percentage identity with known bacterial strains belonged to bacterial genera known to be prolific producers of bioactive compounds and useful extracellular agents. These bacterial genera include *Bacillus* (Mondol et al., 2013), *Vibrio* (Mansson et al., 2011), *Pseudoalteromonas* (Holmström et al., 1999), *Pseudomonas* (Isnansetyo et al., 2009) and *Halomonas* (Biswas et al., 2022).

Several known bacterial species found closely clustered with the isolated bacterial strains in this preliminary study were also reported from various marine sources and are known to produce bioactive molecules. For instance, the heterotrophic *B. altitudinis* has been found associated with marine algae and the sponge, *Haliclona simulans*, and its bacterial extract reported to possess anti proliferative activity against human hepatocellular adenocarcinoma (Phelan et al., 2012). Previous research has shown that a strain of *B. altitudinis*, isolated from marine sediment, can produce antifungal compounds against *Magnaporthe oryzae*, a fungal strain that causes rice blast (Jin et al., 2012). Another strain, *B. vietnamensis*, an endospore forming halotolerant bacterium, was previously reported from Vietnamese fish sauce, sponge and marine sediments (Noguchi et al., 2004). The isolated bacterial strain, S3A22 (Table 2), in this study is most closely related to *Pseudoalteromonas lipolytica* which has the capacity to synthesize bioactive compounds. Strain S3A22, isolated from medium M1, is a slow growing bacterium as its growth was only identified at week 4

and has light cream appearance (Table 2). A brownish pigment, pyomelanin, has been isolated from *P. lipolytica*, whereby its biofilms have displayed anti-larval activity against the settlement and metamorphosis of the mussel *Mytilus coruscus* [83,84]. Strains K3A3 and K3A14, whose closest relatives are *V. neptunius* and *V. alginolyticus*, respectively, are known to produce secondary metabolites (Table 2) (Mansson et al., 2011). Lastly, two known marine bacterial strains, including *Pseudomonas pachastrellae* and *Phaeobacter daeponensis*, are closely related to strains P3A9 and P1B8, respectively (Table 2). The former marine bacterium was previously isolated from a deep-sea marine sponge, *Pachastrella* sp., [87], while *P. daeponensis*, a facultatively anaerobic bacterium, was cultivated from marine sediment (Dogs et al., 2013). Taken together, the isolation of strains belonging to bacterial species/genera known to be prolific producers of bioactive metabolites could suggest their defensive roles in the host sponge, *X. testudinaria*, and conferring host resilience and its widespread occurrence in reef systems. Further ecological-based research is needed to verify the defensive roles of these associated marine bacteria.

Apart from the current study, the microbiome of the local *Xestospongia testudinaria* was previously investigated using culture-independent methods, such as pyrosequencing/amplicon sequencing (Pires et al., 2019). These studies consistently revealed that phylum Chloroflexi was the most abundant microbial group in the host sponge. Previous studies showed that Chloroflexi is one of the most common and diverse bacterial group in sponges with many sponge-specific lineages (Brück et al., 2010). Metagenomic and single-cell genomic approaches to characterize the functional gene repertoire of Chloroflexi symbionts in marine sponges suggested clade-specific metabolic specialization, and that these unique symbionts have the genomic potential for dissolved organic matter degradation from seawater. Moreover, members of the Chloroflexi phylum have the capacity to synthesize bioactive polyketides in marine sponges, suggesting that they could play a role in sponge defence against predators. Other abundant microbial phyla found in local *X. testudinaria* include Crenarchaeota, Acidobacteriota, Proteobacteria and Actinobacteriota. A similar high diversity was found in a pyrosequencing-based analysis of *X. testudinaria* from the Red Sea, where Chloroflexi were also dominant but were followed by Proteobacteria and Firmicutes. A recent study by Deignan and co-workers (2023) showed that the microbiome composition of *X. testudinaria* did not differ across reef sites at Pulau Hantu, Sisters' Islands Marine Park and Kusu Island. Interestingly, their study found significant differentiation in microbiome composition by host sponge *X. testudinaria* genetics in a manner that was aligned with the proposed species boundaries.

Based on phylogenetic analysis, the isolated marine bacterial strains obtained in our study did not belong to any predominant phyla, including Chloroflexi and Acidobacteria, that were detected using culture-independent methods in previous studies. Of the 84 marine bacterial strains obtained in the initial isolation from inoculated marine agar plates, the phylogenetic analysis of 20 microbial strains belonged to either phylum Proteobacteria or Firmicutes. It is possible that the remaining unidentified isolated strains could be members of phyla Chloroflexi and Acidobacteria as difficulties in obtaining good quality DNA material hampered their phylogenetic analysis. Another possible reason could be due to the difficulty in isolating microorganisms of certain microbial phyla, such as Chloroflexi and Actinobacteria, as they are known to be slow growing. Future studies could include longer incubation times (e.g., months) in combination with increasing agar media types and minicolonies detection (Davis et al., 2011). These techniques may prove useful in the isolation of hard to culture microbes from marine sponges to provide further insights into the roles these bacterial groups play in sponge-microbe interactions as well as in biodiscovery efforts.

Conclusions

Of the 84 bacterial strains isolated from the giant barrel sponge, *X. testudinaria*, collected from three different reef systems in Singapore, the phylogenetic analysis of 20 strains were successfully carried out. These bacterial isolates belonged to either phylum Proteobacteria or Firmicutes, consisting of 60% and 40% of the total number of isolated bacterial strains analysed, respectively. In addition, marine bacteria of the genera *Bacillus* and *Vibrio* were found in sponge samples from all three locations, while other bacteria genera, such as *Labrenzia* and *Pseudoalteromonas*, were only successfully isolated from sponge samples from P. Hantu and *Halomonas*, *Marinobacter*, *Pseudomonas* and *Phaeobacter* detected from sponge samples from P. Semakau. Moreover, two bacterial strains, S1B11 and S2A10, showed sequence identity of below 80% with their nearest relative of *Labrenzia alba* and *Bacillus vietnamensis*, respectively, and could constitute new microbial species that warrants further investigation. A majority of the isolated bacterial strains was found clustered in high sequence identity (greater than 97%) with bacterial species known to produce bioactive specialized metabolites. This suggests that these associated microbes could provide chemical defense for the host sponge and contribute to its resilience in Singapore's highly urbanized marine environment. Future work includes increasing the number of media types as well as prolonging the incubation period to isolate other hard to culture bacterial taxonomic groups, such as Chloroflexi and Acidobacteria. This preliminary study on the use of culture-dependent methods can complement culture-independent methods employed by other researchers to better understand the ecological roles and biomedical applications of diverse microbes found in ecologically important *X. testudinaria*.

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