JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

MOLECULAR IDENTIFICATION USING 16S rRNA GENE TO IDENTIFY BACTERIA SYMBIONT-*Agelas* sp. SPONGE WITH ANTIBACTERIAL ACTIVITY

Endang Sulistyarini Gultom[1], Urip Harahap[2], Dwi Suryanto[3], Herbert Sipahutar[1], Rini Hafzari[1]*

Address(es):
[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan, William Iskandar PS. V. Medan, North Sumatera, 20221, Indonesia.
[2] Department of Pharmacy, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, North Sumatera, 20155, Indonesia.
[3] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, North Sumatera, 20155, Indonesia.

*Corresponding author: endanggultom@unimed.ac.id

https://doi.org/10.55251/jmbfs.11840

ARTICLE INFO

Received 23. 9. 2024
Revised 21. 1. 2025
Accepted 21. 1. 2025
Published 1. 2. 2025

Regular article

OPEN ACCESS

ABSTRACT

Sponge (*Agelas* sp.) are complex marine organisms that form symbiotic relationships with bacterial communities. These bacterial symbionts produce bioactive secondary metabolites with various therapeutic potentials, including anticancer, antiviral, anti-inflammatory, and antibacterial effects. This study aims to identify bacterial symbionts associated with *Agelas* sp. sponge from Sibolga, North Sumatera, that have antibacterial activity, using the 16S rRNA gene. The antibacterial activity of these symbionts was evaluated against *Staphylococcus lugdunensis* ESBL, *Pseudomonas aeruginosa* ESBL, and *Klebsiella pneumoniae* ESBL. Ten bacterial isolates exhibiting significant antibacterial activity were identified. Sequencing gen 16S rRNA result showed that these isolates included *Bacillus cereus*, *Pseudomonas aeruginosa*, *Alcaligenes*, and *Vibrio* species, demonstrating efficacy against the tested pathogenic bacteria. The results showed the potential of *Agelas* sp. symbiotic bacteria as a source of natural antibacterial compounds that can be used to develop new antibacterial agents. This study offers a valuable foundation for future exploration of bioactive compounds from sponges with antibacterial applications.

Keywords: Marine Sponge, Symbiont Bacteria, 16S rRNA, Antibacterial, Bioactive Compound

INTRODUCTION

Sea Sponges (Phylum Porifera) are the oldest multicellular organisms, dating back to the Precambrian era. These species are found all over the world and occupy a variety of surfaces in shallow and deep water. They play functional and ecological roles in coral reef systems, such as binding corals to the reef framework and preventing burrowing organisms from entering and destroying them **(Abbas & Mahmoud, 2022)**.

Sponges are a natural product that is known to have a large number of biologically active compounds. They are also responsible for roughly thirty percent of all-natural products derived from marine sources. The biologically active compounds can be used as anticancer, antiviral, anti-inflammatory, immunosuppressive, antibiotic, and other bioactive agents **(Mehbub *et al.*, 2014)**. Sponges are the most prevalent marine invertebrates that engage in symbiosis with bacteria. Over 47 bacterial taxa are symbiotic with sponges, some exclusively associated with them **(Hentschel *et al.*, 2012)**. Besides the considerable microbial diversity in sponges, research has demonstrated that microbial symbionts associated with sponges generate a range of secondary metabolites.

Numerous bioactive chemicals originating from marine sponges indicate structural similarities to metabolites produced by microorganisms. Moreover, metabolites and bioactive compounds sourced from sponge microbes possess significant potential for pharmaceutical and biotechnological applications, rendering them novel targets for the discovery of optimal drug compounds, particularly as antibacterials **(Blunt *et al.*, 2017; Hillman & Goodrich-Blair, 2016)**.

Research by **Gultom *et al.* (2024)** indicates that *Bacillus cereus* symbiotically associates with *Agelas* sp. Sponges generate bioactive chemicals that function as antibacterials against *Klebsiella pneumoniae*. Alternative research *Bacillus cereus* and *Bacillus paramycoides*, symbionts of *Clathrina* sp and *Agelas* sp sponges, have antibacterial efficacy against *Klebsiella pneumoniae* ESBL, *Pseudomonas aeruginosa* ESBL, and *Staphylococcus lugdunensis* MRSA **(Gultom *et al.*, 2021)**. Identifying bacterial species that are symbiotic with sponges is not just important, but it is crucial for elucidating the makeup and function of the microbial community within the sponge, underlining the urgency and significance of our research.

The preliminary identification of bacteria via conventional techniques involves morphological observation, cultivation on selective media, biochemical analysis, and antibiotic susceptibility testing. Despite this, technological advancements have led to the extensive development of molecular technologies for bacterial identification. The choice of particular genes significantly influences the precision of species identification **(Hafzari *et al.*, 2024)**. The gene commonly used to identify microbial species is 16s ribosomal RNA (16S rRNA). This gene is part of 19 protein complexes constituting the 30s subunit of bacterial ribosomes. This subunit is encoded by the highly conserved 16S rRNA gene, essential for ribosome assembly in all bacteria. This subunit also possesses changeable sections that serve as identifiers for specific species. The 16S rRNA gene is an appropriate gene fragment for the identification, comparison, and phylogenetic classification of bacteria **(Johnson *et al.*, 2019)**.

In accordance with the preceding explanation, the objective of this investigation is to identify bacterial species that are symbiotic with marine sponges. Bacterial species that are symbiotic with sponges are anticipated to be developed and considered for cultivation as antibacterial compounds.

MATERIAL AND METHODS

Sample Sponge Collection

The sponge sampling method refers to **(Maarisit et al., 2024)**. A sponge sample was obtained with scuba diving at a water depth of around 10-20 meters in the Sibolga region of North Sumatra Waters. The sample was conducted by mapping the seabed. The sponge was excised with a sterile knife and placed into a sample bag. The sponge was rinsed with sterile seawater, placed in a plastic sample bottle filled with sterile seawater, and stored in a cooler with ice cubes for further investigation at the microbiology laboratory of Medan State University.

Isolation Bacteria Symbiont Sponge

Isolation of Sponge Symbiont Bacteria using the method described by **(Gultom et al., 2021)**. Ten (10) g of sponge samples were cut into pieces and then rinsed twice with sterile seawater to remove the attached particles. The sponge pieces were ground with a mortar and then added with 90 ml of sterile seawater. Dilution was done by taking 1 ml of the stock solution and putting it into a test tube containing 9 ml of sterile seawater. Dilutions were performed 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. One (1) ml of sample was taken from dilutions 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} and inoculated into a petri dish filled with 20 ml of Marine Agar (MA) media, using the spread plate method, then incubated at 37 °C for 24 hours. The morphology of colony cells, namely shape, color, elevation, and edges, was observed after incubation for 24 hours. Colonies with different morphologies were separated by taking the colonies

with a loop, then purified on Marine Agar (MA) medium and incubated at 37 °C for 24 hours.

Antibacterial Activity Test of Sponge Symbiont Bacteria

The sponge symbiont bacteria were transferred into 10 ml of sterile distilled water and dissolved using a vortex. Oxoid disc paper was saturated with 0.1 ml of the sponge symbiont bacterial isolate suspension. A 0.1 ml suspension of the *P. aeruginosa* ESBL isolate, prepared according to McFarland standards, was inoculated onto Muller Hinton Agar (MHA) media using a sterile cotton swab. The paper disc, previously immersed in the sponge symbiont bacterial suspension, was placed on the inoculated media and incubated at 37°C for 24 hours. The inhibition zone, indicated by the clear area surrounding the paper disc, was measured with a caliper **(Gultom et al., 2024)**.

DNA Isolation and Amplification of 16S rRNA Gene of Sponge Symbiont Bacteria with Potential as Antibacterial

The symbiotic bacteria with sponges *Agelas* sp that were found to have antibacterial activity were selected for further DNA extraction and identification using the 16S rRNA gene. DNA was extracted utilizing the Bacteria Iminiprep Kit (Zymo Research, D6005). The entire process of DNA extraction followed with the kit protocol. Amplification was performed utilizing the PCR Gradian Labcycler apparatus (SENSOQUEST). The primers utilized for PCR were the universal primer targeting the 16S rRNA gene. The primers following base sequences: 27F (5'-GAGTTTGATCCTGGCTCAG) and 1492R (5'-GGCTACCTTGTTACGACTT) **(Sadi, 2009)**. The template utilized for 16S rRNA gene amplification was the genomic DNA of sponge symbiotic bacteria, which could exhibit antibacterial properties against pathogenic bacteria. The overall PCR volume was 25 µL, comprising 11.3 µL of nuclease-free water and PCR buffer (10x). 2.5 µL of DNA template; 3 µL of MgCl2 (25 mM); 2.5 µL of primers 27F and 1492R (30 pmol/µL); 0.2 µL of Taq DNA Polymerase (5 U/µL). The PCR protocol consisted of an initial denaturation at 94 ºC for 3 minutes, followed by denaturation at 94 ºC for 1 minute, annealing at 62 ºC for 1 minute, and extension at 72 ºC for 1 minute, repeated for 30 cycles. The PCR results were detected via 1% gel electrophoresis employing SYBR Safe DNA gel staining. Electrophoresis was conducted at a voltage of 75 volts for 60 minutes, utilizing a marker of 100 bp size. The electrophoresis data were analyzed utilizing the UV Light Gel Documentation System (Bio step). The PCR results exhibited distinct bands, followed by sequencing conducted through a Genetics Science, First Base, Singapore service **(Sadi, 2009)**.

Analysis Data

The results of the sequencing of bacterial isolates were then analyzed using the Bioedit 7.2 bioinformatics device and identified using data from the National Center for Biotechnology Information (NCBI) (www.ncbi.nih.nlm.gov) through the BLASTN (Basic Local Alignment Search Tool) program to determine the species of isolate based on the GenBank database. The phylogenetic tree was reconstructed using the MEGA X program by comparing isolates with sequential sequences of the 16S rRNA region obtained from the DNA database at the NCBI GeneBank **(Gultom *et al*., 2021)**

RESULTS AND DISCUSSION

Sponge Identification

Sponge samples collected from Ungge Island, Sibolga, North Sumatera marine waters were submitted to Museum Zoologicum Bogoriense; the voucher specimen was Pori001. Sponges are classified according to distinguishing characteristics per the guidelines of (www.spongeguide.com). The results acquired are as follows:

Figure 1 Sponge *Agelas* sp

The sponge identified in the Sibolga Sea, North Sumatra, at a depth of 10 meters, is *Agelas* sp. Observations indicate that the *Agelas sp* sponge has a tubular or vase-like morphology, a reddish-brown hue, and a dense, rigid texture. **Luissandy *et al*. (2017)** suggest that the *Agelas sp*. sponge belongs to the Demospongiae Class and the Agelasidae Family. This sponge has a huge branched structure made of spongin fibers, exhibiting a hard and uniform texture like stone, located at depths of 3-50 meters on the seabed.

Marine sponges possess a highly diversified microbial life within their tissues. Sponges can host bacteria constituting 40-60% of their total biomass, with densities ranging from 10^8 to 10^{10} bacteria per gram of wet sponge weight. Nonetheless, the sponge symbiont bacteria that have been effectively investigated include less than 1% of taxa. This is due to the disparities in environmental conditions between the ocean and the laboratory **(Asagabaldan *et al*., 2017)**.

The majority of sponges from the four most recent families of Porifera (Demospongiae, Calcarea, Hexactinellida, and Homoscleromorpha) harbor a significant quantity of bacteria and other microorganisms within the mesohyl **(Silva *et al*., 2020)**. Bacteria commonly present in diverse sponges belong to the phyla Actinobacteria, Firmicutes, and Gammaproteobacteria.

The *Agelas robusta* sponge has nine phyla of symbiotic bacteria: Cyanobacteria, Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria, Acidobacteria, Planctomycetes, Bacteroidetes, and Gemmatimonadetes. In total, 30.5% of the clones were categorized inside the genus Synechococcus of Cyanobacteria, while 35% were classified under Proteobacteria. Six distinct bacterial members of *A. robusta* belong to the phyla Proteobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Actinobacteria, and Gemmatimonadetes.

Isolation of Bacteria Symbiont with *Agelas* sp. Sponge Based on Colony Morphology

The findings of isolating the bacteria symbiotic with *Agelas* sp sponge were observed by analyzing the shape, color, edges, and elevation of bacterial colonies developing on MA media. The findings are as follows:

Table 1 Characteristics of Isolate Colonies Bacterial Symbiont with *Agelas* sp

Isolate Code	Shape of Colony	Colony Elevation	Edge of Colony	Colony Colour	Gram
IA1	*Circular*	*Flat*	*Entire*	Beige	-
IA2	*Irregular*	*Flat*	*Serrate*	White	-
IA3	*Circular*	*Convex*	*Entire*	Beige	-
IA4	*Circular*	*Convex*	*Entire*	White	-
IA5	*Irreguler*	*Flat*	*Serrate*	Beige	-
IA6	*Irregular*	*Flat*	*Lobate*	Beige	-
IA7	*Irregular*	*Flat*	*Undulate*	White	-
IA8	*Circular*	*Convex*	*Entire*	Orange	-
IA9	*Rizoid*	*Flat*	*Filiform*	Yellow	-
IA10	*Irregular*	*Raised*	*Entire*	White	-
IA11	*Irregular*	*Raised*	*Undulate*	Red	+
IA12	*Irregular*	*Raised*	*Undulate*	Orange	-
IA13	*Irregular*	*Raised*	*Undulate*	Brown	-
IA14	*Irregular*	*Raised*	*Undulate*	Yellow	-
IA15	*Filamentous*	*Raised*	*Filiform*	Black	-
IA16	*Circular*	*Convex*	*Entire*	Yellow	-
IA17	*Circular*	*Convex*	*Entire*	White	+
IA18	*Filamentous*	*Umbonate*	*Lobate*	Yellow	-
IA19	*Circular*	*Convex*	*Entire*	Yellow	+
IA20	*Circular*	*Convex*	*Entire*	Black	-
IA21	*Irregular*	*Raised*	*Undulate*	Black	-
IA22	*Circular*	*Convex*	*Entire*	Red	+
IA23	*Circular*	*Convex*	*Entire*	White	-
IA24	*Circular*	*Convex*	*Entire*	Black	-
IA25	*Filamentous*	*Flat*	*Filiform*	Orange	-
IA26	*Filamentus*	*Umbonate*	*Filiform*	White	+
IA27	*Circular*	*Flat*	*Entire*	Brown	-
IA28	*Rizoid*	*Raised*	*Filiform*	Orange	-
IA29	*Irregular*	*Flat*	*Entire*	Yellow	-
IA30	*Rizoid*	*Flat*	*Filiform*	White	-

Based on Table 1, the characteristics of the isolate bacteria symbiont with *Agelas* sp sponge are diverse in colony shape, color, edge, and elevation. Six (6) isolates of sponge symbiont bacteria that have a yellow color are IA9, IA14, IA16, IA18, IA19, IA29; Eight (8) isolates are white, namely IA2, 1A4, 1A7, IA10, IA17, IA23, IA26, IA30; four (4) isolates are orange, four (4) isolates are black, two (2) isolates are red and two (2) isolates are brown. The isolates of sponge symbionts also have a variety of shapes. Namely, there are 11 circular isolates, ten irregular isolates, six filamentous isolates, and three rhizoid isolates. The diversity of sponge symbiont isolate elevations was ten raised isolates, nine flat isolates, eight elevated isolates, and three umbonate isolates. A total of 14 isolates had entire colony edges, eight filiform isolates, five undulate isolates, and two lobate isolates.

Antibacterial Activity Of Isolate Bacterial Symbiont With *Agelas* sp Sponge Against Pathogenic Bacteria

Seven isolates of bacteria symbiont with *Agelas* sp sponge demonstrated antibacterial activity against the pathogenic bacteria *K. pneumoniae* ESBL, *P. aeruginosa* ESBL, and *S. lugdunensis* ESBL, categorized as moderate to strong. The results are presented in Table 2. According to Table 2, the findings indicated that 13 isolates of sponge symbiont bacteria exhibited antibacterial potential against *K. pneumoniae* ESBL, categorized as moderate and effective; 17 isolates demonstrated antibacterial potential against *P. aeruginosa* ESBL, classified as weak, moderate, and effective; and 14 isolates showed antibacterial potential against *S. lugdunensis* ESBL, also categorized as weak, moderate, and effective. The isolates IA10, IA17, IA24, and IA7 exhibited modest antibacterial activity against *K. pneumoniae* ESBL and *P. aeruginosa* ESBL. Both bacteria are gram-negative. Isolates IA3, IA4, IA15, and IA29 have antibacterial activity against *P. aeruginosa* ESBL (gram-negative) and *S. lugdunensis* ESBL (gram-positive) within the weak to moderate categories.

Table 2 Antibacterial Activity Test Results of Isolates Bacteria Symbiont with Sponge Against Pathogenic Bacteria

Isolate Code	Inhibition Zone (mm)		
	***K. pneumoniae* ESBL**	***P. aeruginosa* ESBL**	***S. lugdunensis* ESBL**
IA1	0	0	6.25
IA2	0	0	10.25
IA3	0	5.0	8.6
IA4	0	2.0	3.75
IA5	10.65	10.25	9.50
IA6	9.75	8.75	9.37
IA7	11.12	8.87	9.75
IA8	6.50	9.25	8.87
IA9	7.60	6.00	8.00
IA10	9.30	6.00	0
IA11	0	0	0
IA12	8.10	7.80	8.00
IA13	0	0	0
IA14	8.10	8.00	0
IA15	0	7.00	6.70
IA16	8.30	7.00	7.50
IA17	7.50	8.10	0
IA18	0	0	0
IA19	0	6.30	0
IA20	0	0	0
IA21	0	0	7.00
IA22	0	0	0
IA23	0	0	0
IA24	7.30	8.00	0
IA25	7.00	0	0
IA26	0	0	0
IA27	7.40	6.20	0
IA28	0	0	0
IA29	0	6.50	4.32
IA30	0	0	0

Based on the results obtained, ten isolates of sponge symbiont bacteria were selected, which had strong antibacterial activity against *P. aeruginosa* ESBL, *S. lugdunensis* ESBL, and *K. pneumoniae* ESBL, for identification symbiont bacteria using the 16S rRNA method.

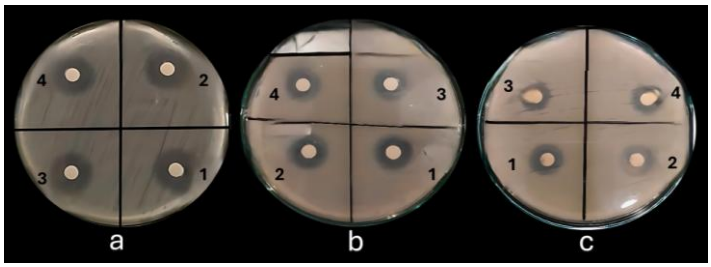

Figure 2 Activity Antibacterial Test
Note: (a) *K. pneumoniae* ESBL, (b) *P. aeruginosa* ESBL and (c) *S. ludgunensis* ESBL, (1) isolate IA5, (2) isolate 1A6, (3) isolate IA7 and (4) isolate IA8.

The antibacterial activity of sponge symbiont bacterial isolates was evaluated against *K. pneumoniae* ESBL, *P. aeruginosa* ESBL, and *S. lugdunensis* ESBL. Out of the 30 isolates collected, only 10 exhibited antibacterial activity against the tested microorganisms. According to **(Esteves et al., 2017),** symbiotic bacteria inhibit microbial growth by producing antimicrobial compounds, which is considered a form of antagonistic activity. The bioactive components comprise steroids, flavonoids, alkaloids, and Micacocidin A, B, and C . Steroids can suppress lactamase enzymes generated by symbiotic bacteria. Flavonoids can directly eliminate bacteria, enhance the efficacy of antibiotics, and diminish bacterial pathogenicity. The alkaloid group inhibits the bacterial nucleic acid synthesis and the dihydrofolate reductase enzyme, a critical enzyme that deactivates antibiotics. Additionally, the deoxygenase enzyme disrupts homeostasis and lyses cells. The Micacocidin Alkaloid group can suppress pathogenic bacterial mycoplasma and function as a chitinase inhibitor, compromising cell walls and damaging the exoskeleton of pathogenic bacteria **(Anand *et al*., 2006; Asagabaldan *et al*., 2017; Gultom *et al*., 2017)**.
Twenty isolates showed no antibacterial activity. Several factors, including environmental adaptation and specialization, may cause this. Many sponge-associated bacteria are highly adapted to their specific host environment and may not produce antibacterial compounds in lab environments, as they do not encounter competition from pathogens like in the wild. Certain compounds may only be produced under environmental stress or specific interactions within the sponge host, which are hard to replicate outside their natural ecosystem. Besides that, secondary metabolite variability also can cause this. Not all sponge-associated bacteria produce antimicrobial compounds consistently. Production of secondary metabolites, including antibiotics, is often regulated by environmental signals and interactions within the sponge's microbiome. Without these cues, some bacteria may not activate the necessary pathways for antibacterial compound production **(Dat et al., 2021; Graça et al., 2015)**

Molecular Identification of 16S rRNA Genes for Potential Bacterial Isolates

The 16S rRNA gene can be utilized to determine bacterial taxonomy, phylogeny (evolutionary relationships), and the spectrum of diversity between species. Furthermore, 16S rRNA gene sequencing is utilized to identify bacteria at the species level and distinguish between closely related bacteria. Ten potential bacterial isolates associated with the sponge *Agelas* sp were successfully sequenced.
The results of the DNA sequencing of sponge symbiont bacteria were analyzed using the Basic Local Alignment Search Tool (BLASTN). The BLAST result analysis provides information and verification regarding bacterial species that have similarities with the DNA sequence of the isolate so that it can be used for bacterial identification. The NCBI blast results can be seen in Table 3
Variations in the inhibition zone might occur from the treatment of sponge symbiont bacteria to gram-negative bacteria, which are notoriously challenging to treat due to their dense and compact peptidoglycan wall and the presence of an efflux pump mechanism. This mechanism expels unnecessary compounds during bacterial cellular biotransformation via the secretion system, thereby impeding the internalization of compounds that could affect bacterial cellular mechanisms **(Wantania et al., 2017)**. Besides that, **Banakar *et al*. (2019)** indicated that bacteria symbiotic with sponges generate secondary metabolites that serve as antibacterials, sourced from the phyla Actinobacteria, Cyanobacteria, Proteobacteria, Firmicutes, and Bacteroidetes, associated with sponges from the genera *Callyspongia*, *Haliclona, Petrosia, Theonella, Dysidea, Xestospongia, Halichondria, Aplysina*, and *Sarcophyton*.
Multiple hit results may be displayed in BLASN results on NCBI. However, the selected results have a high identity value, query coverage, and identity percentage **(Kaur et al., 2008)**. Based on Table 3, it can be seen that the blast sequence results obtained from the sequencing process have homology above 99%. According to **(Stormo, 2009),** if the similarity is ≥ 93%, then it is considered the same species. This also shows that the 16S rRNA gene sequence can be used as a marker of bacterial species that are symbionts with sponges. The 16S rRNA gene is a conserved gene that exists in all organisms. The conserved structure allows the utilization of the 16S rRNA gene in PCR and sequencing studies. This gene's structure has several bases known as hypervariable areas, exhibiting traits that differentiate each creature **(Johnson *et al*., 2019)**.
Bacillus cereus is a bacterium classified to the Firmicutes Phylum, Bacilli Class, Caryopenales Order, and Bacillaceae Family. The length of this bacterial nucleotide is approximately 1482 bp (Sequence Accession Number AF290547), NCBI ID 226900. *Bacillus* spp. It comprises a genus of bacteria that can form symbiotic relationships with sponges, typically residing on their surface **(Verslyppe *et al*., 2014)**. Several Bacillus species synthesize a diverse array of bioactive compounds, such as polyketides, alkaloids, fatty acids, peptides, and terpenes, which exhibit antibacterial, antifungal, and biosurfactant properties **(Dos Santos *et al*., 2015; Freitas-Silva *et al*., 2020)**. *Bacillus pumilus*, isolated from the marine sponge *Petromica citrina*, shown antibiotic efficacy against Gram-negative and Gram-positive infections, including vancomycin-resistant Enterococcus (VRE) strains, multidrug-resistant *Klebsiella pneumoniae*, and multidrug-resistant *Neisseria gonorrhoeae* **(Freitas-Silva *et al*., 2020)**. A *B. subtilis* strain obtained from the sponge *Aplysina aerophoba* synthesized surfactin, iturin, and fengycin, while another *B. subtilis* strain from *Haliclona simulans* produced subtilomycin, both exhibiting bioactivity against *S. aureus* strains **(Phelan *et al*., 2013)**. Pathogens of *B. cereus* obtained from the sponge *Halichondria japonica* synthesize thiopeptide antibiotics effective against ESBL and VRE pathogens.

Vibrio is a genus of the Proteobacteria phylum, Gamma Proteobacteria class, Vibrionales order, and Vibrionaceae family. The nucleotide length is 3400 base pairs with a GC content of 44.77%. This bacterium synthesizes four categories of secondary metabolites: bacteriocin, ectoine, siderophore, and arylpolyne **(Chalasani *et al*., 2015)**. The marine Vibrio genus has been identified as a source of numerous novel chemicals. Over 93 distinct active metabolites have been discovered from Vibrionaceae. It is recognized for generating diverse antibacterial chemicals that ecologically sustain their population in sponges **(Chen *et al*., 2022)**.

Table 3 BLASTN Results From Sponge Symbion Bacteria Sequences

No	Isolate Code	Homology	Accsesion Number	Max Score	Total Score	Query cover	Identity
1	IA5	*Bacillus cereus*	KX941839.1	2001	2001	100%	99,55%
2	IA6	*Pseudomonas aeruginosa*	MT646431.1	2034	2034	100%	99,73%
3	IA7	*Alcaligenes javaensis*	AB914514.1	1035	1128	99%	85,57%
4	IA8	*Alcaligenes aquatilis*	MT572474.1	1011	1718	90%	98,94%
5	IA9	*Alcaligenes aquatilis*	KT748636.1	1711	1711	95%	92,68%
6	IA10	*Pseudomonas aeruginosa*	MT633047.1	1986	1986	100%	99,19%
7	IA12	*Vibrio alginolyticus*	MK102576.1	2161	2161	100%	97,57%
8	IA16	*Vibrio alginolyticus*	MT491126.1	2135	2135	99%	97,82%
9	IA17	*Bacillus cereus*	MN309964.1	1511	1511	100%	99,88%
10	IA24	*Pseudomonas aeruginosa*	KM978038.1	2161	2161	97%	97,49%

Pseudomonas aeruginosa belongs to the Proteobacteria Phylum, Gamma Proteobacteria Class, Pseudomonadales Order, and Pseudomonadaceae Family. *Pseudomonas* sp. is commonly present in several sponge species. This genus is recognized for its antagonistic activities against numerous human pathogenic microorganisms. *Pseudomonas* sp. isolated from *Panova* sp. coral exhibits antagonistic action against the pathogens *E. aerogenes*, *A. baumannii*, *S. aureus*, *E. coli*, and *S. hemolyticus* **(Bakkiyaraj *et al*., 2013)**. *Pseudomonas aeruginosa*, extracted from the Antarctic sponge *Isodictya setifera*, synthesizes six diketopiperazines and two phenazine alkaloids. Phenazine is a pigment with antibacterial effects **(Caruso *et al*., 2022)**.

The phylogenetic tree was constructed to determine the similarities and relationships between nucleotide sequences from potential bacterial isolates associated with the sponge *Agelas* sp. and compare strains on Gene Bank data. The phylogenetic tree reconstruction in Figure 3 indicates that isolates IA5 and IA17 are *Bacillus cereus* species, exhibiting a similarity level of 99.27% to 99.88%. Phylogenetic analysis confirms that isolates IA5 and IA17 belong to the same branch as the *Bacillus cereus* sample retrieved from GeneBank. The phylogenetic tree analysis reveals that isolates IA6, IA10, and IA24 are *Pseudomonas aeruginosa* species, exhibiting a similarity level of 99.19% to 99.73%. The phylogenetic tree corroborates that the three isolates are part of the P*seudomonas aeruginosa* species, occupying the same branch as the *Pseudomonas aeruginosa* sample from GeneBank.

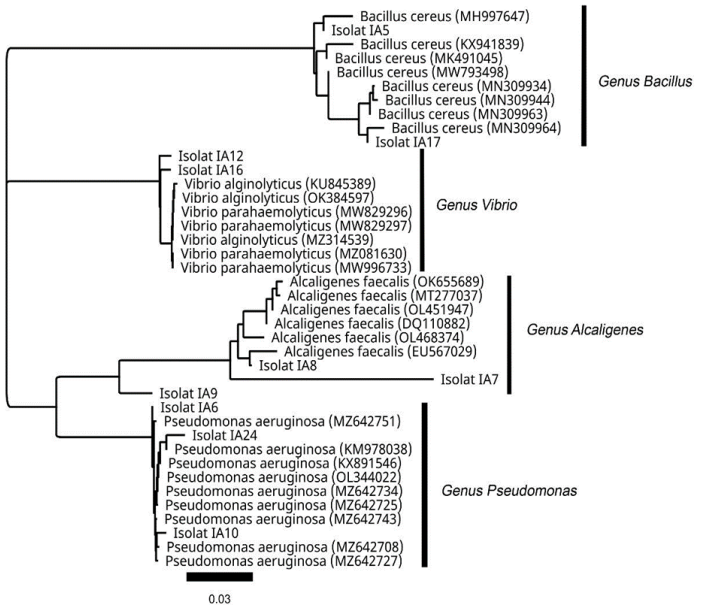

Figure 3 Phylogenetic Tree of Isolates Bacteria Symbiont Sponge *Agela*s sp

Bacillus pacificus belongs to the Firmicutes Phylum, Bacilli Class, Caryophanales Order, and Bacillaceae Family. These bacteria create metabolites such as Acetoin, Hydrogen Sulfide, and Indole and possess enzymes like Arginine dihydrolase, beta-galactosidase, Catalase, Cytochrome Oxidase, Gelatinase, Lysine Decarboxylase, Ornithine Decarboxylase, Tryptophan Deaminase, and Urease. These bacteria are frequently discovered in marine sediments **(Liu *et al*., 2019)**. The bacterial species linked to macroalgae were found to produce α-amylase. A-amylase from B. pacificus may hydrolyze soluble starch, followed by allylose and amylopectin **(Alonazi *et al*., 2021)**.

The phylogenetic tree indicates that the 16S rRNA gene is insufficient for species-level identification of *Alcaligenes* and *Vibrio*. However, it is effective for genus-level differentiation. This is evidenced by isolates IA7, IA8, and IA9, which are classified within various genera of *Alcaligenes*, including *Alcaligenes faecalis*. The isolates IA12 and IA16 are categorized alongside other species of *Vibrio*, including *Vibrio parahaemolyticus* and *Vibrio alginolyticus*; nevertheless, the branches created by the two isolates within the genus diverge substantially. The 16S rRNA marker can distinguish these two isolates at the genus level but is insufficient for species-level identification. **Johnson et al. (2019**) assert that the limitations of the 16S rRNA gene preclude the identification of bacterial species at the species level due to factors such as restricted gene variation and closely related bacterial species that exhibit nearly identical 16S rRNA gene sequences.

CONCLUSION

This study's results offer insights into the bacteria that symbiotically associate with *Agelas* sp. sponges and their antibacterial properties. Among the 30 bacterial isolates, 10 showed antibacterial activity against *Staphylococcus lugdunensis* ESBL, *Pseudomonas aeruginosa* ESBL, and *Klebsiella pneumoniae* ESBL. The sea sponge *Agelas* sp. Harbors bacterial species like *Bacillus, Vibrio, Alcaligenes,* and *Pseudomonas* exhibit antimicrobial properties, promoting further investigation to discover novel antimicrobials. This study screened only representative isolates for activity, as not all strains could be separated due to capacity constraints. Consequently, subsequent research should examine more strains and characterize prospective novel antimicrobials, including evaluating their cytotoxicity.

REFERENCES

Abbas, S., & Mahmoud, H. (2022). Identification of Sponge-Associated Bacteria From the Coast of Kuwait and Their Potential Biotechnological Applications. *Front Microbiol*, *13*, 896718. https://doi.org/10.3389/fmicb.2022.896718

Alonazi, M., Karray, A., Badjah-Hadj-Ahmed, A. Y., & Ben Bacha, A. (2021). Alpha Amylase from *Bacillus pacificus* Associated with Brown Algae *Turbinaria ornata*: Cultural Conditions, Purification, and Biochemical Characterization. *9*(1), 16. https://doi.org/10.3390/pr9010016

Anand, T. P., Bhat, A. W., Shouche, Y. S., Roy, U., Siddharth, J., & Sarma, S. P. (2006). Antimicrobial activity of marine bacteria associated with sponges from the waters off the coast of South East India. *Microbiological Research*, *161*(3), 252-262. https://doi.org/10.1016/j.micres.2005.09.002

Asagabaldan, M., Ayuningrum, D., Kristiana, R., Sabdono, A., Radjasa, o. k., & Trianto, A. (2017). Identification and Antibacterial Activity of Bacteria Isolated from Marine Sponge *Haliclona* (Reniera) sp. against Multidrug Resistant Human Pathogen. *IOP Conference Series: Earth and Environmental Science*, *55*, 012019. https://doi.org/10.1088/1755-1315/55/1/012019

Bakkiyaraj, D., Sivasankar, C., & Pandian, S. K. (2013). Anti-pathogenic Potential of Coral Associated Bacteria Isolated from Gulf of Mannar Against *Pseudomonas aeruginosa*. *Indian J Microbiol*, *53*(1), 111-113. https://doi.org/10.1007/s12088-012-0342-3

Banakar, R., Eggenberger, A. L., Lee, K., Wright, D. A., Murugan, K., Zarecor, S., . . . Wang, K. (2019). High-frequency random DNA insertions upon co-delivery of CRISPR-Cas9 ribonucleoprotein and selectable marker plasmid in rice. *Scientific Reports*, *9*(1), 19902. https://doi.org/10.1038/s41598-019-55681-y

Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H. G., & Prinsep, M. R. (2017). Marine natural products [10.1039/C6NP00124F]. *Natural Product Reports*, *34*(3), 235-294. https://doi.org/10.1039/c6np00124f

Caruso, G., Papale, M., Azzaro, M., Rizzo, C., Laganà, P., Caruso, R., & Lo Giudice, A. (2022). Antarctic Porifera homogenates as a source of enzymes and antibacterial substances: first results. *Polar Biology*, *45*(5), 895-907. https://doi.org/10.1007/s00300-022-03042-3

Chalasani, A. G., Dhanarajan, G., Nema, S., Sen, R., & Roy, U. (2015). An Antimicrobial Metabolite from *Bacillus* sp.: Significant Activity Against Pathogenic Bacteria Including Multidrug-Resistant Clinical Strains. *Front Microbiol*, *6*, 1335. https://doi.org/10.3389/fmicb.2015.01335

Chen, L., Wang, X.-N., Bi, H.-Y., & Wang, G.-Y. (2022). Antimicrobial Biosynthetic Potential and Phylogenetic Analysis of Culturable Bacteria Associated with the Sponge *Ophlitaspongia* sp. from the Yellow Sea, China. *20*(10), 588. https://doi.org/10.3390/md20100588

Dat, T. T. H., Steinert, G., Cuc, N. T. K., Smidt, H., & Sipkema, D. (2021). Bacteria Cultivated From Sponges and Bacteria Not Yet Cultivated From Sponges—A Review [Review]. *12*. https://doi.org/10.3389/fmicb.2021.737925

Dos Santos, I. P., da Silva, L. C., da Silva, M. V., de Araújo, J. M., Cavalcanti Mda, S., & Lima, V. L. (2015). Antibacterial activity of endophytic fungi from leaves of Indigofera suffruticosa Miller (Fabaceae). *Front Microbiol*, *6*, 350. https://doi.org/10.3389/fmicb.2015.00350

Esteves, A. I., Cullen, A., & Thomas, T. (2017). Competitive interactions between sponge-associated bacteria. *FEMS Microbiol Ecol*, *93*(3). https://doi.org/10.1093/femsec/fix008

Freitas-Silva, J., Silva-Oliveira, T., Muricy, G., & Laport, M. S. (2020). *Bacillus Strains* Associated to *Homoscleromorpha* Sponges are Highly Active Against Multidrug Resistant Bacteria. *Curr Microbiol*, *77*(5), 807-815. https://doi.org/10.1007/s00284-019-01870-x

Graça, A. P., Viana, F., Bondoso, J., Correia, M. I., Gomes, L., Humanes, M., . . . Lage, O. M. (2015). The antimicrobial activity of heterotrophic bacteria isolated from the marine sponge *Erylus deficiens* (Astrophorida, Geodiidae) [Original Research]. *6*. https://doi.org/10.3389/fmicb.2015.00389

Gultom, E., Suryanto, D., Munir, E., & Diningrat, D. (2017). Bacteria Extract Activity Associated With Sponges *Haliclona* sp.2 *Andaxinellid* Sp.As Antibacterial. *International Journal of Advanced Research*, *5*, 751-759. https://doi.org/10.21474/ijar01/2810

Gultom, E. S., Harahap, U., Suryanto, D., Sipahutar, H., & Restuati, M. (2024). Antibacterial Activity of Ethanol Extract of the Marine Sponge (*Agelas* sp) Symbiont *Bacillus cereus* MH997647 IA5 against *Klebsiella pneumoniae* ESBL. *Tropical Journal of Natural Product Research*, *8*(1). https://doi.org/10.26538/tjnpr/v8i1.37

Gultom, E. S., Hasruddin, Sitompul, A. F., Situmorang, A. D., & Prasetya, E. (2021). Identifying Sponge Symbiont Bacterial with Antibacterial Activity against Multidrug Resistant Organism (MDRO) Bacteria from Sea Waters in Sibolga, North Sumatra Indonesia. *Biosfer: Jurnal Tadris Biologi*, *12*(2), 169-184. https://doi.org/10.24042/biosfer.v12i2.10138

Hafzari, R., Annisa, Anita, K., Muchamad Nur, C., Listya Puspa, K., Nurul Huda, P., . . . Dwi Ratna Anjaning Kusuma, M. (2024). Precision And Reliability Of Nanoplate Digital PCR System For Pork DNA Identification And Quantification. *Journal of microbiology, biotechnology and food sciences*, *14*(1), e10691. https://doi.org/10.55251/jmbfs.10691

Hentschel, U., Piel, J., Degnan, S. M., & Taylor, M. W. (2012). Genomic Insights Into The Marine Sponge Microbiome. *Nature Reviews Microbiology*, *10*(9), 641-654. https://doi.org/10.1038/nrmicro2839

Hillman, K., & Goodrich-Blair, H. (2016). Are You My Symbiont? Microbial Polymorphic Toxins And Antimicrobial Compounds As Honest Signals Of Beneficial Symbiotic Defensive Traits. *Current Opinion in Microbiology*, *31*, 184-190. https://doi.org/10.1016/j.mib.2016.04.010

Johnson, J. S., Spakowicz, D. J., Hong, B.-Y., Petersen, L. M., Demkowicz, P., Chen, L., . . . Weinstock, G. M. (2019). Evaluation of 16S rRNA Gene Sequencing For Species And Strain-Level Microbiome Analysis. *Nature Communications*, *10*(1), 5029. https://doi.org/10.1038/s41467-019-13036-1

Kaur, H., Amandeep, S., & Singh, P. (2008). Comparison of Variants of BLAST. *Lecture Notes in Engineering and Computer Science*, *2168*.

Liu, J., Meng, Z., Liu, X., & Zhang, X.-H. (2019). Microbial Assembly, Interaction, Functioning, Activity And Diversification: A Review Derived From Community Compositional Data. *Marine Life Science & Technology*, *1*(1), 112-128. https://doi.org/10.1007/s42995-019-00004-3

Luissandy, L., Deiske, S., & Rosita, L. (2017). Bioaktivitas Antibakteri Fraksi ODS Spons *Agelas* SP. Dari Perairan Pulau Bunaken. *Jurnal Pesisir dan Laut Tropis*, *2*(1), 22-30. https://doi.org/10.35800/jplt.5.3.2017.16936

Maarisit, W., Pareta, D. N., Lengkey, Y. K., & Untu, S. D. (2024). Anti-Mycobacterial Activity Of Secondary Metabolites From Marine Sponge *Agelas* sp. *Tropical Journal of Natural Product Research (TJNPR)*, *8*(10), 8883 - 8888. https://doi.org/10.26538/tjnpr/v8i10.33

Mehbub, M. F., Lei, J., Franco, C., & Zhang, W. (2014). Marine Sponge Derived Natural Products between 2001 and 2010: Trends and Opportunities for Discovery of Bioactives. *12*(8), 4539-4577. https://doi.org/10.3390/md12084539

Phelan, R. W., Barret, M., Cotter, P. D., O'Connor, P. M., Chen, R., Morrissey, J. P., . . . Barbosa, T. M. (2013). Subtilomycin: A New Lantibiotic From *Bacillus subtilis* Strain MMA7 Isolated From The Marine Sponge *Haliclona simulans*. *Mar Drugs*, *11*(6), 1878-1898. https://doi.org/10.3390/md11061878

Stormo, G. (2009). An Introduction to Sequence Similarity ("Homology") Searching. *Current protocols in bioinformatics / editoral board, Andreas D. Baxevanis ... [et al.]*, *Chapter 3*, Unit 3.1 3.1.1-7. https://doi.org/10.1002/0471250953.bi0301s27

Verslyppe, B., De Smet, W., De Baets, B., De Vos, P., & Dawyndt, P. (2014). Strain Info Introduces Electronic Passports For Microorganisms. *Systematic and Applied Microbiology*, *37*(1), 42-50. https://doi.org/10.1016/j.syapm.2013.11.002

Wantania, L. L., Ginting, E. L., & Wullur, S. (2017). Isolasi Bakteri Simbion Dengan Spons Dari Perairan Tongkeina, Sulawesi Utara. *Jurnal Lppm Bidang Sains Dan Teknologi*, *3*(1), 57-65.