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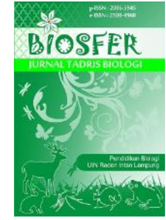

Identifying Sponge Symbiont Bacterial with Antibacterial Activity against Multi-Drug Resistant Organism (MDRO) Bacteria from Sea Waters in Sibolga, North Sumatra Indonesia

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ABSTRACT

This research employed the experimental method with descriptive data analysis. It aimed to isolate and identify sponge symbiont bacteria with antibacterial potential against Multi-Drug Resistance Organisms (MDRO) with the 16S rRNA gene marker. Based on the study results, two sponges were obtained, namely *Clathrina* sp and *Agelas* sp, each having 11 and 7 isolates of symbiont bacteria, respectively. Based on the antibacterial activity test results, out of 18 isolates of symbiont bacteria, only 4 had antibacterial potential against MDRO (*Klebsiella pneumoniae* ESBL, *Pseudomonas aeruginosa* ESBL, or *Staphylococcus lugdunensis* MRSA) with inhibition zones of 9.3 mm, 8.3 mm, and 8.1 mm, respectively. The results of bacterial identification using the 16S rRNA gene sequence isolate S1I3, S1I9, and A1I3 belong to the *Bacillus cereus* and *Bacillus paramycoides* types. This research is expected to provide important information about sponge symbiont bacteria with antibacterial potential against MDRO.

Identifikasi bakteri simbion spons sebagai antibakteri terhadap bakteri Multi Drug Resistant Organism (MDRO) asal perairan laut Sibolga, Sumatera Utara Indonesia

ABSTRAK: *Tujuan penelitian ini adalah untuk mengisolasi dan mengidentifikasi bakteri simbion spons yang berpotensi sebagai antibakteri terhadap Multi Drug Resistance Organism (MDRO) dengan penanda gen 16S rRNA. Berdasarkan hasil penelitian, diperoleh 2 spons yaitu Clathrina sp dan Agelas sp, masing masing memiliki 11 dan 7 isolat bakteri simbion. Berdasarkan hasil uji aktifitas antibakteri, dari 18 isolat bakteri simbion, hanya 4 isolat bakteri yang berpotensi sebagai antibakteri terhadap MDRO (Klebsiella pneumoniae ESBL, Pseudomonas aeruginosa ESBL, atau Stapyhlococcus lugdunensis MRSA) dengan zona hambat masing-masing 9,3 mm, 8,3 mm, dan 8,1 mm. Hasil identifikasi bakteri menggunakan sekuen gen 16S rRNA, isolat S1I3, S1I9, dan A1I3 termasuk ke dalam jenis Bacillus cereus dan Bacillus paramycoides. Penelitian ini diharapkan memberikan informasi penting tentang bakteri simbion spons yang berpotensi sebagai antibakteri MDRO.*

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INTRODUCTION

Diseases caused by bacterial infections are among the most common diseases in Indonesia (Dirga et al. 2021). Various synthetic antibiotics have been developed as a therapy for bacterial infectious diseases. Still, the irrational use of synthetic antibiotics can lead to resistance without a doctor's prescription and non-continuously (Yenny and Herwana 2007). The development of antibiotic resistance is an urgent problem because many types of pathogenic bacteria are reported. They are resistant to more than two types of antibiotics (Aarestrup 1999) or commonly referred to as *Multi-Drug Resistance Organism* (MDRO) (Harmawan, Ridho, and Pringgenies 2012; Barie 2012; Bowler et al. 2012).

Many pieces of research on MDRO bacteria have been carried out, namely *Staphylococcus aureus* against methicillin; *Pseudomonas aeruginosa* against piperacillin, ceftazidime (Okunye 2012), aztreonam, imipenem, ciprofloxacin, and aminoglycosides; *Enterobacteriaceae* against β-lactam class antibiotics; *Klebsiella pneumoniae* against aminoglycosides and ciprofloxacin (Dwiprahasto 2005); *Salmonella typhi* against cotrimoxazole, chloramphenicol, ampicillin, and amoxicillin (Sandika and Suwandi 2017). The *Eschericia coli* bacteria is also resistant to fluroquinolones and cotrimoxazole (Qadri et al. 2005). *Bacillus cereus* 5/B line (ATCC 13061) is resistant to penicillin (Fenselau et al. 2008), Ceftazidime, and Ciprofloxacin (Fisch et al. 2012). *Bacillus subtilis* is resistant to *ciproflox, norfloxacin, gentamycin, Amoxil, streptomycin, rifampicin, erythromycin, chloramphenicol, ampiclox, azithromycin, ceftriaxone, carbenicillin*, and *levoflox* (Ekpo et al. 2017; Naik et al. 2010).

The search for bioactive compounds that can produce antimicrobial compounds of natural origin is needed because of the increasing cases of pathogenic microorganisms' resistance to drugs currently used in general. It drives the need for new types of antibiotics (Manikandan et al. 2011). The diversity of marine life can be used as a source of bioactive compounds that have the potential as an antibacterial agent (Rasyid 2008). Research on symbiotic bacteria with sponges has attracted the attention of researchers because it has been proven to contain active compounds that can be used as raw ingredients for antibiotic drugs. (Aboul-Ela et al. 2019; Restuati and Gultom 2012; Gultom et al. 2017; Tinambunan, Melki, and Isnaini 2012). Previous studies have shown that symbiotic bacteria with sponges can inhibit the growth of pathogenic bacteria (Hoppers et al. 2015), such as the sponge symbiont bacteria called *Haliclona simulans* against Gram-positive (Indraningrat, Smidt, and Sipkema 2016) and Gram-negative pathogenic bacteria (Phelan et al. 2013); *Plakortis* sp (Pasodung et al. 2018), *Dictyonella funicularis* (Ngantung, Sumilat, and Bara 2016), *Phyllospongia lamellose* (Ngantung, Sumilat, and Bara 2016) from the waters of Bunaken Island and *Siphonodictyon* sp., *Ircinia* sp., *Dysidea* sp. sponges from the waters of Salibabu in the Talaud Islands (Maradou et al. 2019) against the *Staphylococcus aureus* and *Eschericia coli* bacteria; *Aplysina aerophoba* sponge against diarrhea-causing bacteria *Helicobacter pylori, Shigella dysenteriae* (Fajrina, Bakhtra, and Irenda 2018), and *Xestospongia testudinaria* sponge against *Staphylococcus aureus* (Fristiohady et al. 2021)*, Pseudomonas aeruginosa*, *Eschericia coli,* and *Salmonella typhi* bacteria (Cita et al. 2017); *Chondrilla caribensis* sponge against *Staphylococcus aureus* (Pech-Puch et al. 2020), *S epidermidis,* and *Escherichia coli* bacteria (Marques et al. 2018).

Identification of bacteria is generally made by phenotypic and genotypic methods (Nurhayati et al. 2011). The phenotypic method is carried out based on physiological and biochemical activity. On the other hand, genotyping is carried out based on

molecular activity by looking at the results of DNA sequencing using the Polymerase Chain Reactions (PCR) method (Ammor et al. 2005; Nurhayati et al. 2011). The weakness of the phenotypic method is the low level of reproducibility (Duarte, Freitas, and Bexiga 2015) and the many procedures and steps that lead to a long processing time. Furthermore, the identification results are less accurate because they rely on phenotypic expressions rendering them prone to misidentification (Chenoll, Macián, and Aznar 2003). When compared with the phenotypic method (Akihary and Kolondam 2020), the genotypic method using the 16S rRNA gene is more advantageous because of its reproducibility (Robeson et al. 2020), shorter processing steps and time, and the ability to distinguish between species more accurately to avoid misidentification (Akihary and Kolondam 2020; Tejesvi et al. 2007).

In the sea waters of Sibolga (Yusni and Uliya 2019), some sponges have antibacterial activity (Restuati and Gultom 2012). However, the type of bacteria is not yet known. Therefore (Reynolds and Thomas 2016), this research was conducted to identify bacteria that live in symbionts with sponges in Sibolga (Murniasih et al. 2021) sea using a molecular-based method (Chitrampalam and Nelson Jr 2014). The identification is made by analyzing the 16S rRNA gene sequencing for its high level of accuracy. Based on this research, three isolates have antibacterial activity against Staphylococcus aureus (Srinivasa Reddy et al. 2001) and Escherichia coli (Kim et al. 2011).

METHOD

a. Sponge Symbiont Bacteria Isolation

Sponge sampling was carried out via scuba diving at a sea depth of ± 10-20 m in the Sibolga waters of North Sumatra. The sponge was then extracted using sterile seawater and diluted to 10^{-1},10^{-2},10^{-3}, 10^{-4},10^{-5}. The dilution results were then planted into Zobell marine Agar media using the pour cup method. The bacterial colonies that grew were identified, where the different bacterial colonies were separated, or the bacterial isolates were purified with Zobell Marine Agar media using the tilted media method.

b. Antibacterial Activity Test on Sponge Symbiont Bacteria Isolates

The isolated sponge symbiont bacteria obtained were then taken one ose and transferred to 10 ml of sterile distilled water and dissolved using a vortex mixer. Oxoid disc paper was soaked for 10-15 minutes in a sponge symbiont bacterial isolate suspension. Suspension of MDR bacterial isolates (*Pseudomonas aeruginosa* ESBL, *Klebsiella pneumoniae* ESBL, and *Staphylococcus lugdunensis* MRSA) was transferred to a sterile cotton bud, left for 30 seconds, then smeared on the surface of the NA media so that it was evenly distributed. Oxoid discs that have been soaked in a suspension of sponge symbiont bacteria were placed on the surface of the inoculated media. The MDR bacterial isolates were then incubated at 35^0C- 37^0C for 24 hours. Furthermore, the zone of inhibition, namely the clear area around the oxoid disc, was measured using a caliper. The three most potential isolates were selected by looking at the bacterial isolates with the largest transparent zone.

c. Molecular Identification of Sponge Bacterial Isolates

The bacterial DNA isolates were carried out using the Bacteria I miniprep Kit (Zymo Research, D6005). The researchers obtained three isolates with the most potential from the isolates of sponge symbiont bacteria. They were selected by looking at the value of the largest inhibition zone. The isolates were identified molecularly using sequences generated from the 16S rRNA region. 16S rRNA amplification was performed using two universal primers Forward 63 F (5'-CAG GCC

TAA CAC ATG CAA GTC-3') and Primer Reserve 138R (5'-GGG CGG TGT GTA CAA GGC-3') (Mahulette and Kurnia 2020).

The amplification of the 16S rRNA fragment was carried out for 30 cycles with each stage of denaturation at a temperature of 95ºC for 5 minutes, annealing at a temperature of 55ºC for 1 minute, elongation at a temperature of 72ºC for 1.5 minutes, and extension at a temperature of 72ºC for 10 minutes (Mahulette and Kurnia 2020). The PCR products were visualized by 1% gel electrophoresis using SYBR Safe DNA gel staining. Electrophoresis was carried out with a voltage of 75 volts for 60 minutes, and the marker used had a size of 100 bp. The electrophoresis results were observed using the UV Light Gel Documentation System (Bio step). PCR results showed clear bands followed by sequencing using a sequencing service from Genetics Science, First Base, Singapore.

d. Data Analysis

The results of the sequencing of bacterial isolates were then analyzed using the Bioedit 7.1.3 bioinformatics device and identified using data from the National Center for Biotechnology Information (NCBI) (www.ncbi.nih.nlm.gov) through the BLAST (*Basic Local Alignment Search Tool*) program to determine the type 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.

RESULTS AND DISCUSSION

a. Sponge Symbiont Bacteria Isolates Activity Test

Sponge samples obtained from the sea waters of Sibolga, North Sumatra, consisted of two types, namely *Clathrina* sp. and *Agelas* sp., identified using www.spongeguide.uncw.edu. Based on the research results, eighteen isolates of sponge symbiont bacteria were obtained from the sponge *Clathrina* sp. and *Agelas* sp. Each sponge had eleven and seven isolates, respectively. The results of the antibacterial activity test against eighteen isolates of sponge symbiont bacteria resulted in four isolates (C1I3, C1I5, C1I9, and A1I3) which had antibacterial potential against *Multi-Drug Resistance Organism* (MDRO) *Klebsiella pneumoniae* ESBL with each inhibition zone formed being 9.3mm, 4.5mm, 8.3 mm, and 8.1 mm respectively. It was classified as moderate activity. There were three isolates (C1I1, C1I3, and C1I10) with antibacterial potential against *Pseudomonas aeruginosa* ESBL with inhibition zones of 6 mm, 7.9 mm, and 6.3 mm, respectively. They were classified as medium resistance. The isolates that had the antibacterial potential to *Staphylococcus lugdunensis* MRSA were three in total (C1I3, C1I6, and A1I3), with inhibition zones of 7mm, 6.7mm, and 7mm, respectively. They were classified as medium resistance. The antibacterial activity of sponge symbiont isolates against MDRO is presented in Table 1.

Table 1. Antibacterial Activity of Sponge Symbiont Bacteria Isolates against MDRO

Bacterial isolate	Obstacles Zone (mm)		
	KP	PA	SL
C1I1	-	6	-
C1I2	-	-	-
C1I3	9.3	7.9	7
C1I4	-	-	-
C1I5	4,5	-	-
C1I6	-	-	6.7
C1I7	-	-	-
C1I8	-	-	-
C1I9	8.3	-	-
C1I10	-	6.3	-
C1I11	-	-	-
A1I1	-	-	-
A1I2	-	-	-
A1I3	8.1	-	-
A1I4	-	-	-

A1I5	-	-	7
A1I6	-	-	-
A1I7	-	-	-

KP: *Klebsiella pneumoniaee* ESBL; PA: *Pseudomonas aeruginosa* ESBL; SL: *Staphylococcus lugdunensis* MRSA

According to Muscholl-Silberhorn, Thiel, and Imhoff (2008), bacteria that are symbiotic with the sponge Clathrina clathris and have antibacterial activity are Alphaproteobacteria (AF218241), Gammaproteobacteria (AY563032), and AJ561154. Clathrina aurea sponge is symbiotic with the bacterium Pseudovibrio ascidiaceicola F423 (EU862084) (Santos et al. 2010), which effectively inhibits bacterial growth of Staphylococcus aureus, S. epidermidis, S. haemolyticus, S. hominis, Enterococcus faecalis, Enterococcus faecium, E. coli 54AE, S. enterica, Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Corynebacterium fimi, and Stenotrophomonas maltophilia (Indraningrat, Smidt, and Sipkema 2016). Also, according to (Santos-Gandelman et al. 2013), the sponge Clathrina aurea is symbiotic with bacteria Bacillus sp., Kocuria sp., and Pseudovibrio sp., which can inhibit the growth of gram-negative bacteria. Bacteria in symbiosis with Clathrina sp. has the antibacterial ability because the sponge Clathrina sp. contains alkaloid compounds in Clathridin and 2-aminoimidazole, which can inhibit bacterial growth (Roué et al. 2010; Roué et al. 2012).

Based on a study by Indraningrat et al. (2019), symbiotic bacteria with sponge Agelas sventres are Actinobacteria, Synechococcus Pseudovibrio, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria types. Bacteria in symbiosis with the sponge Agelas sp. has antibacterial activity against Staphylococcus aureus and Escherichia coli (Luissandy, Sumilat, and Lintang 2017). Bacteria in symbiosis with Agelas sp. has the antibacterial ability because of a study by (Undap, Sumilat, and Bara 2019). The thin-layer chromatography (TLC) test with bioautography technique in Agelas sp. found the presence of terpenoid and flavonoid compounds which have antibacterial activity against Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, S. saprophyticus, and Escherichia coli. Agelas dispar has longamide B and keramadine compounds (Cafieri, Fattorusso, and Taglialatela-Scafati 1998). Agelas nakamurai contains ageliferine, debromosceptrin, and nakasiswa acid compounds (Eder et al. 1999), which have antibacterial activity against B. subtilis, S. aureus dan E. coli.

The Results of DNA Amplification of Sponge Symbiont Bacteria Isolates That Have Antibacterial Activity against MDRO Bacteria

Three isolates of sponge symbiont bacteria that had the most potential to have antibacterial activity were bacteria coded C1I3, C1I9, and AII3. These bacteria were then extracted and amplified using 16 rRNA and electrophoresis. The visualization results show a band with a size of ±1300 bp parallel to ±1350 bp (Figure 1). These results are then sequenced to determine the type of bacterial symbionts.

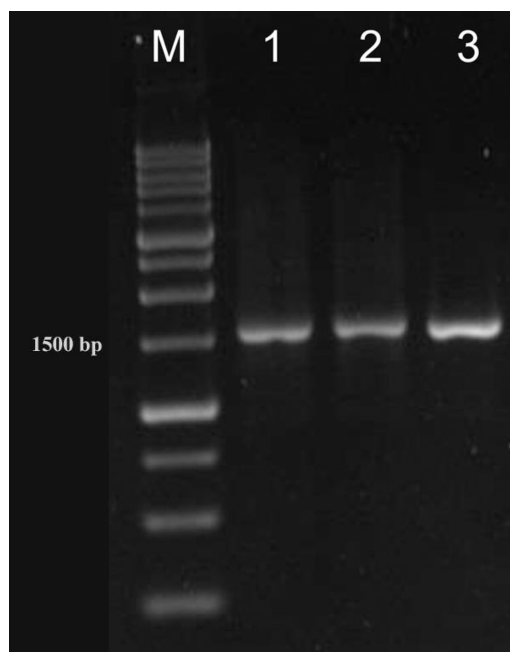

Figure 1. Electrophoresis Visualization of Gene 16S rRNA Isolates 1(C1I3), 2 (C1I9), and 3 (A1I3)

Based on the results of 16rRNA sequencing conducted at PT. Genetic Science Indonesia, the C1I3 isolate was 984 bp, the C1I9 isolate was 1018 bp, and the A1I3 isolate was 993 bp. The nucleotide sequences obtained from each isolate were analyzed using BLAST. The results of the BLAST analysis of C1I3 bacterial isolates based on Genbank data are: Bacillus cereus (MT611946.1) with a similarity level of 100%; C1I9, based on Genbank data, is included in Bacillus paramycoides (MT538529.1) with 100% similarity level; and A1I3, based on Genbank data, is included in the Bacillus paramycoides (MW148452.1) with 100% similarity level.

The 16S rRNA gene can see the similarity of each species to an accuracy of 99% (Janda and Abbott 2007; Akihary and Kolondam 2020; TRIANTO et al. 2019). According to Harwood and Wipat (2006), if an individual's similarity is 93%, then it is considered the same species. However, according to Riesenfeld, Schloss, and Handelsman (2004), if the maximum similarity is 97%, it will already be considered as a single species. According to Bosshard et al. (2003), in identifying gram-positive bacteria using 16rRNA, if the similarity between individual bacteria is $<$ 95%, then the bacteria would belong to a single family if the similarity is $\geq$ 95% $- <$ 99% is considered as belonging to the same genus and if it's $\geq$ 99 % the bacteria would be considered the same species. Based on the similarity results obtained, the three isolates of sponge symbiont bacteria with antibacterial activity are Bacillus cereus (C1I3) and Bacillus paramycoides (C1I9 and A1I3).

After the species of bacterial isolates were known, a phylogenetic tree was made. The phylogenetic tree has branches connecting each species, while the root of the tree is the signature of the parent individual of the entire organism being analyzed (Felix et al. 2011; Alawiyyah, Budiharjo, and Suprihadi 2017). The phylogenetic tree aims to show the kinship relationships of each species tested based on various observed characteristics, both morphological and molecular (PANGESTIKA et al. 2015). Based on the data obtained from Genbank for each species and individual comparison, a phylogenetic tree was created (Figure 2).

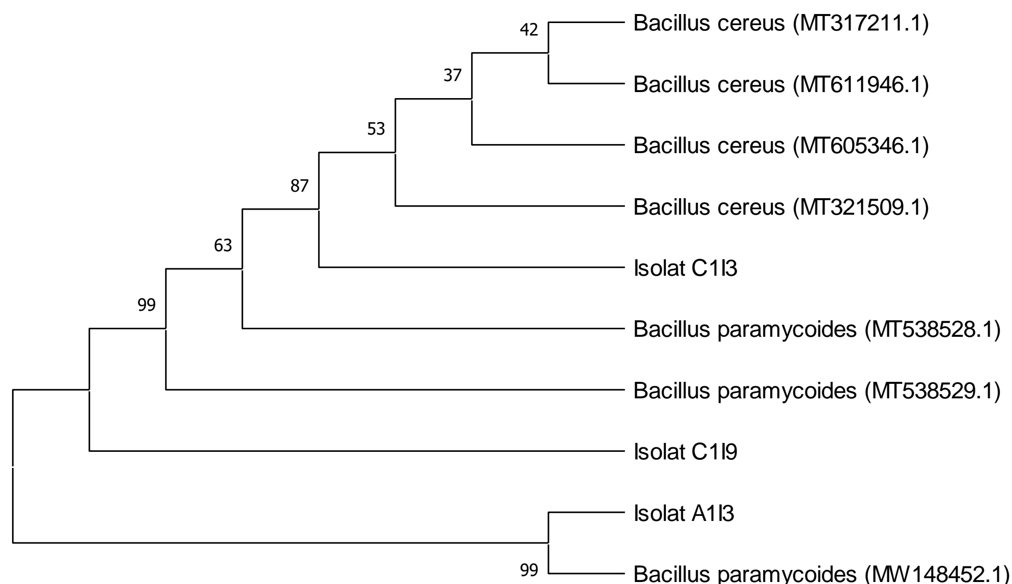

Figure 2. Phylogenetic tree Showing the Relationship between Sample Isolates and Other Bacteria from GeneBank based on 16S rRNA Sequences

Based on the phylogenetic tree analysis in Figure 2, it can be seen that isolates C1I3, C1I9, and A1I3 belong to the bacteria genus *Bacillus*. Isolate C1I3 was close to *Bacillus cereus*, while isolates C1I9 and A1I3 belonged to the type of *Bacillus paramycoides* bacteria. *Bacillus* is a genus widely distributed in many habitats both on land and in water (Ivanova et al. 1999; Y. Liu et al. 2013), including marine sediments (Miranda, Martins, and Clementino 2008; Ettoumi et al. 2009). Miranda, Martins, and Clementino (2008) found 23 isolates of *Bacillus* consisting of 4 species, namely *B. subtilis, B. licheniformis, B. cereus*, and *B. pumilus*, in various marine sediments of the Brazilian ocean.

Ki, Zhang, and Qian (2009) found 13 species of *Bacillus*, among which are *B. cereus* BU040901-020, *B. anthracis* UST2006-BC001, *B. aquamarines* NC_006510UST040801-016, *B. badius* UST2006-BC002, *B. firmus* UST991130-006, *B. halmapalus* UST981101-001, *B. hwajinpoensis* UST040801-008, *B. litoralis* UST2006-BC006, *B. sporothermodurans* UST2006-BC007, *B. vietnamensis* UST040801-005, *B. asahii* CU040510-015, *B. seohaenensis* UST2006-BC008, and *B. lentus* UST991130-010 in various sediments from the oceans in Hong Kong. In addition, various studies that have utilized bacteria from the *Bacillus* genus have also been carried out by (Chopra et al. 2014), who isolated *B.sonorensis* as a new antibiotic in the form of sonorensin from soil samples in the Parangipettai ocean, India. Hu, Wang, and MacMillan (2011) isolated the bacteria *B. hunanensis* from the Bahamas mangrove swamps known to produce the antibiotic hunanamycin. The *Bacillus cereus* QN03323 Strain was extracted from *Halichondria japonica* whose two new substances belong to the thiopeptide class of antibiotics (SUZUMURA et al. 2003). The sponge *Dysidea fragilis* is symbiotic with the

bacteria *Bacillus* sp., which produces secondary metabolites and has broad-spectrum antibacterial activity (Mohan, Thipparamalai Thangappanpillai, and Ramasamy 2016).

Molecular identification can distinguish bacteria from the genus Bacillus with a fairly large genetic distance value (Ki, Zhang, and Qian 2009; Rodiansyah et al. 2021). The smaller the genetic distance value between species, the closer the relationship between the two species (Subari, Razak, and Sumarmin 2021). The genetic distance between isolates of bacterial samples was about 0.000-0.002, while with the genus Bacillus, the genetic distance was 0.000-0.009 (Table 2).

Table 2. Genetic Distance of Sampled Bacterial Isolates with the Genus *Bacillus*

No	Sample and Species	1	2	3	4	5	6	7	8	9
1	Isolat_C1I3									
2	*Bacillus cereus* (MT317211.1)	0.000								
3	*Bacillus cereus* (MT321509.1)	0.000	0.000							
4	*Bacillus cereus* (MT605346.1)	0.000	0.000	0.000						
5	*Bacillus cereus* (MT611946.1)	0.000	0.000	0.000	0.000					
6	Isolat C1I9	0.002	0.002	0.002	0.002	0.002				
7	Isolat A1I3	0.002	0.003	0.002	0.002	0.002	0.000			
8	*Bacillus paramycoides* (MT538528.1)	0.001	0.002	0.001	0.001	0.001	0.000	0.000		
9	*Bacillus paramycoides* (MW148452.1)	0.009	0.008	0.007	0.007	0.007	0.001	0.000	0.006	
10	*Bacillus paramycoides* (MT538529.1)	0.001	0.002	0.001	0.001	0.001	0.000	0.000	0.000	0.006

The 16rRNA gene has several advantages in identifying bacteria, namely (1) In every organism, the 16rRNA gene is identical in ubiquity (its presence is always maintained under any conditions); (2) If there is an evolution in an organism, the 16SrRNA gene can indicate the evolutionary distance so that it can be used as an evolutionary chronometer; (3) The 16S rRNA gene has a conservative and varied base sequence that can construct a universal phylogenetic tree in reflecting the evolutionary chronology of an organism; (4) Has a hypervariable region section that helps in identifying the species of a bacterium (PANGASTUTI 2006). A study by (Cihan et al. 2012) used the 16rRNA gene to identify bacteria of the *Bacillus* genus growing in geothermal areas in Turkey; the results showed that the similarity of the *Bacillus* genus was between 94-100%. *Bacillus paramycoides* bacteria have a close relationship and can be included in the *Bacillus cereus* group of bacteria by 93-99,6% (NISA et al. 2021; Fatwa, Yoswaty, and Effendi 2021; Carroll, Wiedmann, and Kovac 2020; Y. Liu et al. 2017). Therefore, the genetic distance of the sample bacteria with *Bacillus cereus* and *Bacillus paramycoides* bacteria in this study was very small, namely 0.000-0.009.

CONCLUSION

The isolates of sponge symbiont bacteria had the most antibacterial potential against the test bacteria *Klebsiella pneumoniae* ESBL, *Pseudomonas aeruginosa* ESBL, and *Staphylococcus lugdunensis* MRSA were C1I3, C1I9, and AII3 isolates. 16S rRNA gene sequencing was adapted to the GenBank database using the BLAST method. It was found that the bacterial isolates that had antibacterial activity were the *Bacillus cereus* (C1I3) and *Bacillus paramycoides* (C1I9; A1I3), with a similarity level above 98%.

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