Identification and Antibacterial Activity of Bacteria Isolated from Marine Sponge *Haliclona (Reniera)* sp. against Multi-Drug Resistant Human Pathogen

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Abstract. The marine sponge *Haliclona (Reniera)* sp. was a potential source of natural bioactive compounds. This sponge widely distributed along the coast of Panjang Island, Jepara, Indonesia. The aims of this research were to isolate the associated bacteria with *Haliclona (Reniera)* sp. and to screen the antibacterial activity against Multi-Drug Resistant (MDR) bacteria. Amount five bacteria were isolated using media selective for bacteria. The antibacterial activities of bacteria were performed by overlay methods. The bacteria strain PSP. 39-04 had the best activity against *Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii,* and *Enterobacter cloaceae.* Based on colony morphology and phylogenetic characterization using 16S rRNA gene sequencing, PSP 39-04 was closely related with *Chromohalobacter salixigens strain DSM3043.*

Keywords: *Haliclona (Reniera)* sp., bacteria, screening, Multi-Drug Resistant, antibacterial

1. Introduction

Infectious disease agents such as *Pseudomonas aeruginosa, Klebsiella pneumonia, Escherichia coli, Staphylococcus aureus, Acinetobacter baumannii,* and *Enterobacter cloaceae*, became a serious health problem in many developing countries, including in Indonesia [1],[2],[3], because they led into morbidity and high mortility in humans [4]. In recent years, those pathogen bacterial became into organisms resistant to multiple antibiotics, or also called the Multi Drug Resistant (MDR). The increasing number of victims have been reported [5],[6],[7]. Moreover, these problems led to higher maintenance costs and only a few hospitals that could solved this problems [4]. The emergence of discovery the newer antibiotics were needed with a promise for combating MDR strains [8],[9],[10].

Many studies have been reported that the natural resources from the sea have abundant potential bioactive compounds against human pathogens [11],[12],[13],[14],[15]. Sponge had into the richest sources bioactive compounds and could be used in the field of pharmacy [16],[17],[18],[19],[20]. Since more than 50 years, sponge organism had been attracted the attention of researchers because of its ability

to produce the natural bioactive compounds that derived from the secondary metabolisms [13]. Several studies have been reported that the sponge *Haliclona (Reniera*) sp. produced Haliclonin-A, as the antibacterial compounds caused by human disease bacterial [21]. In addition, the compounds was from sponge *Haliclona crassiloba* has been reported to inhibit the activity of pathogenic bacteria in humans both negative and positive grams [14]. Other studies have shown that extracts of Sponge *Haliclona* sp. had some activity against gram-positive bacteria *Staphylococus aureus*, and *Bacillus subtilis* [15].

However, the main problems was the limitations of the sponge organisms. Exploration of bioactive compounds from sponge required a lot of biomaterial that might happened *over-exploitation* and low levels of conservation [22],[23]. Bacteria associated with sponge was a new breakthrough for searching the bioactive compounds, because it was easily produced with rapid and large scale, without having to take excessive sponge [24],[25]. Sponge became one of the residences of marine microorganisms where located in mesohil layer of sponge or commonly called *High Microbial Abundance Sponge* (HMAS) including bacteria. Role of bacteria in the sponge was as a defense from predators [26] and also helped in the metabolism of a sponge [27],[28]. Another important role of the bacteria associated with the sponge has produced bioactive compounds which acted as antimicrobial, antiviral, antioxidant and antitumor activity [19,29,30]. Several studies have shown that extracts of some bacteria associated with sponge *Haliclona vansoesti* have antibacterial activity against human diseases such as *S. aureus, P. aeruginosa, E. coli* and *B. subtilis* [31]. The sponge *Cliona* sp. associated bacteria *Comamonas testosteroni* and *Citrobacter freundii* hade a compound that can be used as a candidate against Ebola virus [32].

In the present study, we identify the associated bacteria isolated from sponge *Haliclona (Reniera)* sp. from Panjang Island, Indonesia and determine the antibacterial activity against MDR human pathogen bacteria.

2. Matherial and Methods

2.1. Sample collection of sponge

The sponge *Haliclona (Reniera)* sp. (figure 1) were collected from Teluk Awur (06˚36'57.4"S, 110˚38'19.4"E) and Panjang Island (06˚34'35.2"S, 110˚37'52.7"E), Jepara, North Java, Indonesia by snorkling from a depth of approximately 3 m. The collection sponge were put into sterile plastic bags underwater to avoid contact with air and brought to Laboratory of Tropical Marine Biotechnology, Diponegoro University. The Specimens were processed under aseptic conditions as follows: sponge were rinsed with sterile seawater and 1 cm^3 of sponge tissue was excised from the middle of the whole sponge with sterile knife. The sponge were macerated at room temperature (RT, 24 ± 2ºC) for 5 min.

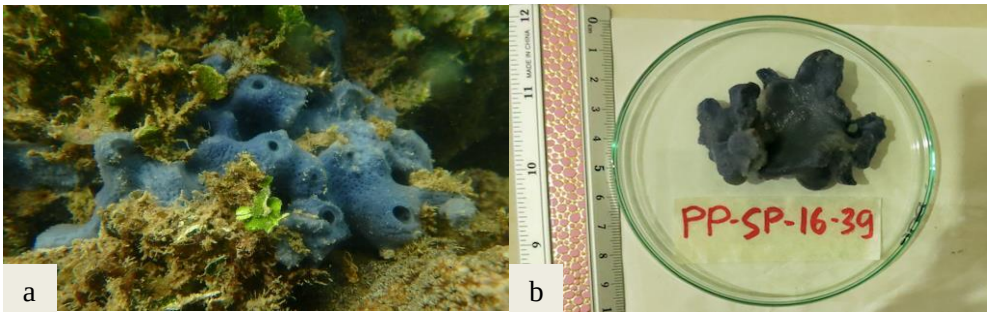

Figure 1. (a) selected Sponge *Haliclona (Reniera)* sp. from Panjang Island, Jepara; (b) sample collection (PP-SP-16-39) was under aseptic conditions

2.2. Isolation and purification of bacteria

The macerated sponge were serially diluted, spread on ½ strenght Zobell 2216E marine agar medium [33],[34],[35] and incubated at room temperate (RT, 24 ± 2ºC) for 48 h. On the basis of morphological features, colonies were randomly picked and purified by making streak plates [36]. The strains of bacteria associated were named with the initials of sponge from which they were isolated. To purify the bacteria isolates, the colonies with different morphological characteristics were picked up and transferred onto freshly prepared media until pure cultures were obtained.

2.3. Screening for antibacterial activities against MDR pathogen bacteria

Screening biological activity of bacteria associated with sponge were conducted by testing against isolate pathogenic bacteria *P. aeruginosa, K. pneumonia, E. coli, S. aureus, A. baumannii,* and *E. cloaceae,* that were obtained from Kariadi Hospitals, Semarang. A total of five bacteria isolates from sponge were tested by using overlay methods [36]. Those isolates of bacteria were inoculated in agar medium and were incubated at optimum temperate growth for bacteria (37 ± 2ºC) for 18 – 20 h. On the other sides, the isolates of MDR pathogenic bacteria were inoculated in soft agar medium (70% agar compotition) with the concentration were 0,5 McFarland, then it were poured into the surface of bacteria isolates (after incubation). The plates were incubated at optimum temperate growth for bacteria (37 ± 2ºC) for 24 h. The antibacterial activity of MDR pathogen was defined by the formation of Diameter of Inhibitation (DOI) around the bacteria isolates.

2.4. Morphological and biochemical characterization of associated bacteria

Morphological characterization of potential bacteria isolates was performed by gram staining according to method previously described [37]. Biochemical tests (indole, oxidase, urease, Citrate Simmons, nitrate reduction, lysine decarboxylase, maltose, and sucrose) were carried out at Laboratory of Tropical Marine Biotechnology and Microbiology Laboratory, Medical Faculty, Diponegoro University.

2.5. Amplification of partial 16S ribosomal RNA of associated bacteria

The molecular-based works were carried out at Laboratory of Tropical Marine Biotechnology, Diponegoro University. Genomic DNA of potential bacteria isolates was used as a template for polymerase chain reaction (PCR) amplification. The amplification of 16S ribosomal RNA (rRNA) was performed using primers 27f (5′-AGAGTTT- GATCMTGGCTCAG-3′) and 1492r (5′-TACGGY-TACCTTGTTACGACTT-3′) [38]. The PCR was conducted in thermal cycler (Perkin – Elmer, USA) under the following cycling conditions according to methods that previously described [36][37], it consisted of an initial denaturation at 95 ºC for 3 min and then 30 cycles with denaturation at 94 ºC for 1 min, annealing at 55 C for 1 min, and extension at 72 ºC for 1 min. A final extension was performed at 72 ºC for 7 min. The PCR products were analyzed by agarose (1%) gel electrophoresis and the digital images were obtained using UVI Doc HD5 (UVITEC Cambridge).

2.6. DNA sequencing and Phylogenetic analysis

The DNA sequencings were carried out by Molecular Biology Laboratory, Agency for the Assesment and Application Technology (BPPT), Jakarta, Indonesia. To identify the closest neighbors of potential bacteria isolates, the 16S rRNA gene sequences were analyzed using BLAST [40]. The 16S rRNA gene sequences were aligned and a neighbor-joining phylogenetic tree was constructed using MEGA 6 according to methods that previously described by Tamura *et al.* [41]. Bootstrap tests were performed 1,000 times using MEGA 6.

3. Results and discussion

3.1. Isolation of Reniera sp. associated bacteria

The associated bacteria were obtained from three dilution series of macerated sponge samples. The results are shown in table 1. Only five cultured bacteria were associated with *Reniera* sp. with different morphological characteristics.

Table 1. Morphological characteristics of *Reniera* sp. associated bacteria

No.	Isolates	Colour	Size	Shape	Margin	Elevation
1	PSP.39-01	White	Small	Round	Entire	Convex
2	PSP.39-02	White	Pin point	Round	Entire	Convex
3	PSP.39-03	White	Small	Round	Entire	Convex
4	PSP.39-04	Red	Small	Round	Entire	Convex
5	PSP.39-05	Opaque yellow	Small	Round	Entire	Convex

3.2. Screening of anti-MDR bacterial activities

Associated bacteria of *Reniera* sp. were tested antibacterial activities against MDR Pathogens. Only the isolates bacteria with code PSP.39-04 shown the positive antibacterial activities against *P. aeruginosa* (21.20 ± 0.26 mm), *E. cloaceae* (17.13 ± 0.31 mm), *A. baumannii* (15.90 ± 0.26 mm), and *S. aureus* (13.10 ± 0.26 mm) (figure 2) and shown negative against *K. pneumonia* and *E. coli.* The results are shown in table 2.

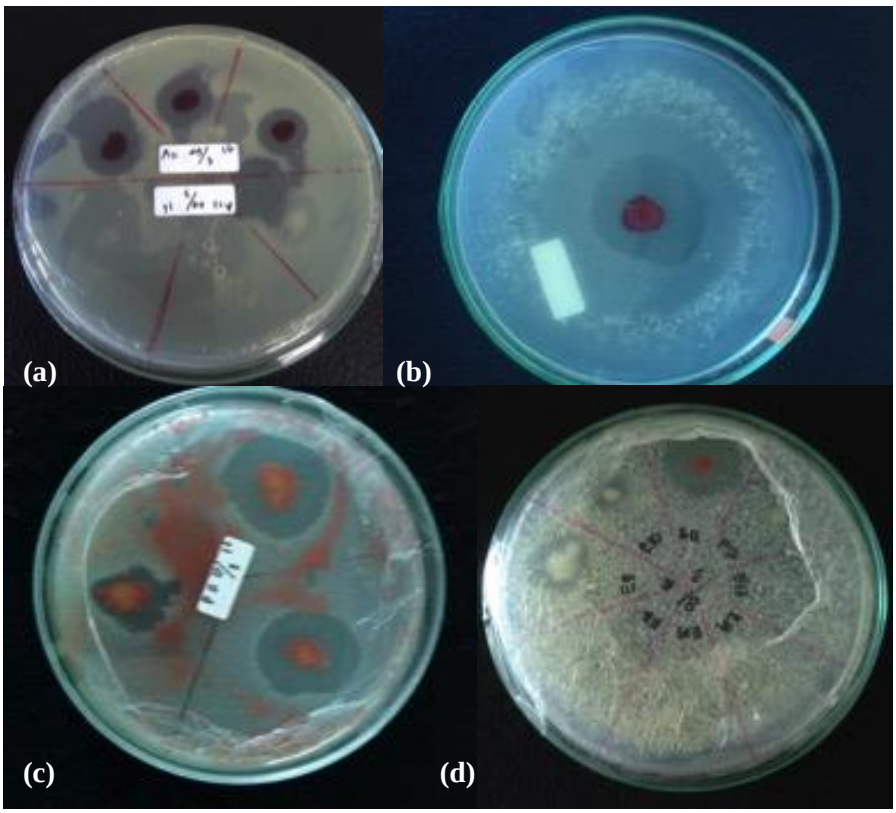

Figure 2. The antibacterial activity of PSP.39-04 isolated bacteria against MDR pathogen; (a) against MRSA *S. aureus,* (b) against MDR *E. cloaceae,* (c) against MDR *P. aeruginosa,* (d) against MDR *A. baumannii*

Table 2. Screening of *Reniera* sp. associated bacteria against MDR pathogen bacteria

Isolates	Zone of Inhibition (mm)					
	P.aeruginosa	*K. pneumonia*	*E. cloaceae*	*A. baumannii*	*E. coli*	*S. aureus MRSA*
PSP-39-01	-	-	-	-	-	-
PSP-39-02	-	-	-	-	-	-
PSP-39-03	-	-	-	-	-	-
PSP-39-04	21.20 ± 0.26	-	17.13 ± 0.31	15.90 ± 0.26	-	13.10 ± 0.26
PSP-39-05	-	-	-	-	-	-

3.3. Morphological and biochemical characterization of PSP.39-04 bacteria isolates

The morphology of isolated bacteria PSP.39-04 was belonged to gram negative (red colour) bacteria and rod shapes, that shown in figure 3.

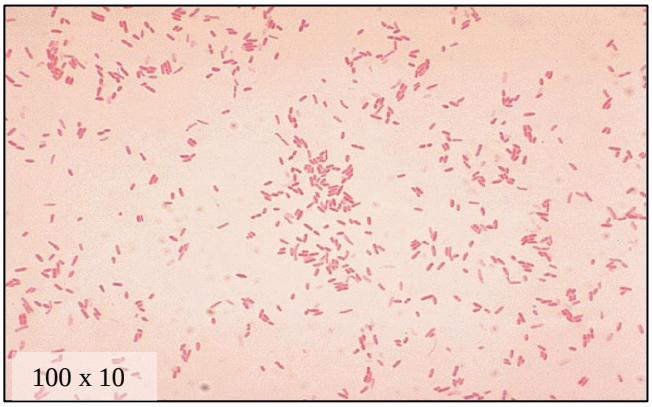

Figure 3. The morphological caracterization of PSP.39-04 bacteria isolates with 100 x10 magnification

The result of biochemical characterization shown that PSP.39-04 isolates in table 3.

Table 3. The biochemical characterization of PSP.39-04 bacteria isolates from *Reniera* sp.

No.	Biochemical characteristics	Tests
1	Oxidase	-
2	Nitrate reduction	+
3	Anaerobic growth with nitrate	+
4	Urease	+
5	Indole	-
6	Citrate (Simmons)	+
7	Hydrolysis of casein	+
8	Ornithine decarboxylase	-
9	Lysine decarboxylase	-
10	Arabinose	+
11	Maltose	+
12	Sucrose	-
13	d –Trehalose	+
14	H5S production	+
15	d-Cellobiose	-
16	Sucrose	+

3.4. Amplification of partial 16S ribosomal RNA of PSP.39-04 bacteria isolates

Amplification and gel electrophoresis of 16S rRNA showed that PSP.39-04 bacteria isolates had approximately 1500bp and it was belonged to bacteria groups. PSP.39-04 DNA amplification were be able to do the sequencing of DNA. The results of 16S rRNA analysis shown in figure 4.

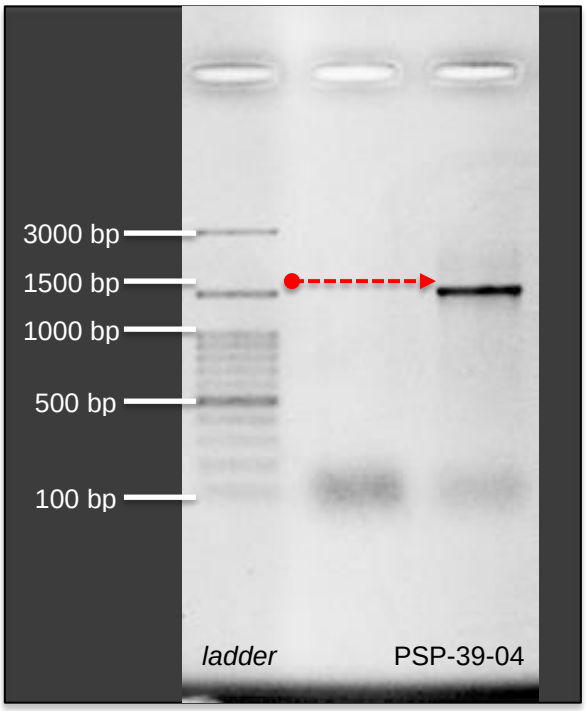

Figure 4. The 16S rRNA analysis of PSP.39-04 bacteria isolates

3.5. DNA sequencing and Phylogenetic contructions of PSP.39-04 bacteria isolates

Based on DNA sequencing, the DNA amplification of PSP.39-04 Isolates represented around 1376bp and it shown in figure 5.

CAGTCGAGCGGTAACAGGTCCAGCTTGCTGGACGCTGACGAGCGGCGGACGGGTGAGTAATGCATAGGAATCTACCCAGTCGTGGGGGATAAC
CTGGGGAAACCCAGGCTAATACCGCATACGTCCTACGGGAGAAAGCGGGGGCTCTTCGGACCTCGCGCGATTGGATGAGCCTATGTCGGATTA
GCTGGTTGGTGGGGTAACGGCTCACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCTTTCGGGTTGTAAA
GCACTTTCAGTGGGGAAGAAGGCTTGTCGGCCAATACCCGGCAAGAGCGACATCACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGCTTGTCACGCCGGGTGTGAAAGCCCCGGGC
TCAACCTGGGAACGGCATCCGGAACGGGCAGGCTAGAGTGCAGGAGAGGAAGGTAGAATTCCCGGTGTAGCGGTGAAATGCGTAGAGATCGG
GAGGAATACCAGTGGCGAAGGCGGCCTTCTGGACTGACACTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGTCGACTAGCCGTTGGGTCCCTTGAGGACTTAGTGGCGCAGTTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCC
GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACCCT
TGACATCCTGCGAACCCGGAAGAGATTCCGGGGTGCCTTCGGGAGCGCAAAGACAGGTGCTGCATGGCTGTCCTCAGCTCGTGTTGTGAAATGT
TGGGTTAAGTCCCGTAACGAGCCTAACCCTTGTTCCTATTTGCCAGCGATTCGGTCGGGAACTCTAGGGAAACTGCCGGTGACAAACCGGAGGA
AGGTGGGGACAACTTCAAGTCATCATGGCCCTTACGGGTAGGGCTACACACGTGCTACAATGGCCGGTACAAAGGGTTGCGAAGCCGCGAGGT
GAAGCCAATCCCAGAAAGCCGGCCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGCATCAGAATGG
CACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGACTGCACCAGAAG

*A=Adenin; G=Guanine; T=Timin; C=Cytosin

Figure 5. The DNA sequencing results of PSP.39-04 isolates

The construction of phylogentic trees was analysed with BLAST homology and the PSP.39-04 isolates was closely related with *Chromohalobacter salexigens* strain DSM 3043 as much as 99%, Halomonadaceae. The phylogenetic trees of PSP.39-04 isolates shown in figure 6.

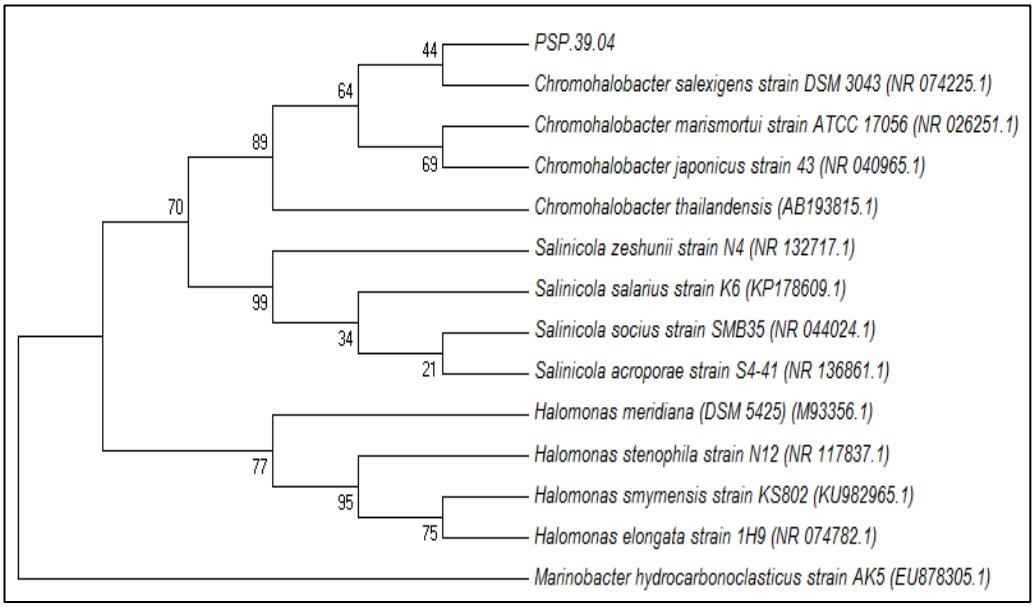

Figure 6. The phylogenetic trees of PSP.39-04 isolates

The antimicrobial activity of sponge *Reniera* sp. had been done in 1979 with Renierone [42] and in 2007, Renierosides [43] as antimicrobial metabolites. We assume that secondary metabolites were derived to associated bacteria, according to study previously reported [44],[45],[46],[47],[48]. The presence of sponge plays as host to high density of microorganisms (virus, bacteria, fungi) or as known as High Microbial Abundance Sponge (HMAS) with 40-60% of biomass of sponge consists of microorganisms. We reported only five bacteria can be isolated in sponge samples (*Reniera* sp.). The results was high contrast with the nature of sponge which high density of microbial. This is due to of the characteristic of associated bacteria, that isolation of bacteria just used one culture media (Zobell) where it is a non-specific or non-selective media. The sponge associated bacteria have been investigated that less than 1% of taxa are be able to culture under laboratory conditions [49]. Hence, we can use a wide range of culture condition approaches in next study.

Screening of Antibacterial activity is the one of useful and effective ways for the preliminary assessment to know which associated bacteria have antibacterial activity. Thus, we are able to keep focus on it. The present study reported that the associated bacteria of sponge *Haliclona (Reniera)* sp. from Pulau Panjang, Indonesia showed high antibacterial activity against MDR pathogen. Only the isolates of PSP.39-04 showed strong growth inhibition of MDR-pathogen *P. aeruginosa, A. baumannii, E. cloaceae,* and *E. coli.* The growth inhabitation was performed by the role of secondary metabolites from PSP.39-04, which will be active when the presence of MDR-pathogens.

The pathogenic bacteria *P. aeruginosa, E. cloaceae, A. baumannii,* and *E. coli* are gram negatives bacteria, while *S. aureus* is gram positive which resistant with methicillin groups (MRSA). The secondary metabolites of PSP.39-04 isolates can inhibit the activity of gram negatives bacteria which have lipopolysaccharides (LPS) cell walls and we assume it have inhibitor for beta lactamase enzymes were produced by *S. aureus.* The previous research by Radjasa *et al.* [34] investigated that the associated bacteria of *Haliclona* sp. had an antimicrobial activities against MDR-pathogen. Another research showed the sponge *Haliclona* sp. associated bacteria have antibacterial activities against pathogenic

bacteria [19],[48],[50],[51]. This result offers using [52] sponge *Haliclona* sp. associated bacteria as the source of antibacterial compounds for controlling the MDR-pathogenic bacteria.

The phylogenetic tree analysis showed that PSP.39-04 bacteria isolates is closely related to *Chromohalobacter salexigens strain DSM3043* which isolated from solar salt facilities [53]. It is a halophilic or salt needed for growth and displays a high halotolerant, as the ability survive in wide range of salt concentration [54]. Thus, from this feature, *C. salexigens* has special metabolites that perform for to survive. *C. salexigens* is included in Gammaproteobacteria that produces many bioactive compounds against MDR-pathogens [55],[56],[57],[58] and. Genus Chromohalobacter also produces halo beta lactamase enzymes and efflux pump that can excrete toxic compounds from cells [59]. The recent study showed that *Chromohalobacter* sp. produced bioactive compounds (cyclic peptides) against MDR pathogens [60] and another research was done by making *Chromohalobacter* sp. as biogenic to synthesize nanoparticles for combating against MDR pathogens [61]. We assume that PSP.39-04 bacteria isolates from sponge *Haliclona (Reniera)* sp. is the potential bacteria for developing new bioactive compounds against MDR-pathogens.

4. Conclusion

In this present study, we revealed that the marine sponge *Haliclona (Reniera)* sp. associated bacteria with PSP.39-04 codes performed the antibacterial activity against MDR pathogenic bacteria, which are *P. aeruginosa, E. cloaceae, A. baumannii,* and *S. aureus (MRSA)*. The PSP.39-04 was closely related to *Chromohalobacter salexigens* with 99% homology. It could be a vital source for promoting as antibacterial. Hence, the sponge *Haliclona (Reniera)* sp. associated bacteria is considered as a good source for the isolation of bioactive compounds and development of new drugs against MDR pathogens.

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