

BACTERIAL DIVERSITY OF THE GORGONIAN CORAL *Plexaura* sp.: SCREENING FOR ANTI-PATHOGENIC PROPERTY AGAINST NOSOCOMIAL PATHOGENIC *Acinetobacter baumannii*

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Abstract

Chronic infectious diseases caused by biofilm-forming pathogenic bacteria are the second rank causes of death in Indonesia after heart and blood vessel diseases. The emergence of antibiotic-resistant *Acinetobacter baumannii* pathogenic bacteria worldwide poses the main medicinal challenge to the search for new antibiotics. However, the use of marine invertebrates as raw material sources is not ecologically friendly due to the low concentration of bioactive compounds in marine invertebrates. This study aimed to investigate gorgonian *Plexaura* sp.-associated bacteria that have the potential as anti-nosocomial pathogenic *A. baumannii*. A total of 29 gorgonian *Plexaura* sp.-associated bacterial isolates were screened for their antibacterial activity against nosocomial pathogenic *A. baumannii*. The 5 bacterial isolates exhibited antipathogenic activity against *A. baumannii*. The RA17-2 isolate showed the highest diameter size of the inhibition zone. The 16S rRNA sequence analyses revealed that these 5 isolates were closely related to *Bacillus*, *Virgibacillus*, and *Nitratireductor*. None of the 5 antipathogenic isolates possess PKS-I, PKS-II, and NRPS genes, except the PKS-I gene of the RA17-2 isolate. These results showed that *Plexauridae* sp is a potential source for the development of antibiotic drugs.

Keywords: Gorgonian; Antipathogen; Biofilm-forming infectious pathogens; *Plexauridae* sp

Introduction

Acinetobacter is one of the bacteria that cause nosocomial infections in the world. In the past, these bacteria were considered low-virulence pathogens, but are now recognized as important disease-causing agents of nosocomial infections [1]. These low-virulence organisms have turned into multidrug-resistant pathogens and are of great concern to healthcare providers around the world. All over the world, 10% of hospitalized patients develop a new infection during their stay, and 1.4 million are infected each year [2]. In Indonesia alone, cases of *Acinetobacter* infection are 25.8%, of which 80% of these infections are due to *Acinetobacter baumannii* [3]. These bacteria are widely reported as causative agents of nosocomial infections in the urinary tract, surgical wound infections, and blood vessel infections [4, 5]. Many studies reported that *A. baumannii* rapidly develops antimicrobial resistance that effectively escapes the effects of antibacterial drugs [6-8]. Because few effective antibiotics are available, doctors often face challenges when treating patients with multi-drug-resistant *A. baumannii* (MDRAB). To address this problem effectively, a possible strategy that can be used to inhibit the spread of

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antimicrobial resistance is antibiotic treatment. However, due to the current unsatisfactory therapeutic results, there is a great need to develop and evaluate the efficacy of new antibiotics.

Marine invertebrates such as sponges, tunicates, bryozoans, and gorgonians are the source of structurally unique natural products. This high marine biodiversity have the potential materials as the source of unique chemical compounds for the development of pharmaceutical, nutraceutical, and cosmetic industries. Arborek is a small island located in the pristine area of the east part of the Indonesian sea, a low, flat island, and is at risk of inundation from sea-level rise. This island is famous for the huge schools of Manta rays and also for the marvelous corals. There is good snorkeling from almost anywhere on the island, but the gorgonian octocorals, i.e., *Plexaura sp.* around the island's eastern end occur at their greatest diversity (Fig. 1).



Fig. 1. *Plexaura sp.* of Arborek Island, Raja Ampat, West Papua

Plexaura sp. is unique among gorgonians due to its prominence and worth as the commercial source of pharmaceutically active compounds. Some previous studies reported that the genus *Plexaura sp.* is one of the gorgonian species known to have biotechnology potential to produce antibacterial and antioxidant active compounds [9, 10]. Pawlik [11] indicated that *Plexaura sp.* were potent anti-predatory defenses against fish. Further, Hunt [12] reported that gorgonian *Pseudoplexaura* and *Pseudopterogorgia* demonstrated the highest inhibitory activity against nosocomial pathogenic bacteria (*S. aureus* (MSSA), *E. coli*, and *P. aeruginosa*).

However, the problem is the supply of raw materials due to the low concentration of bioactive compounds in marine invertebrates. As illustrated, obtaining 1 g of anti-cancer compounds required marine tunicate as much as 1 metric ton wet weight [13]. On the other hand, only 10 square centimeters of gorgonian is needed for the source of bacterial isolates. It means that the use of marine invertebrates as raw material sources is not ecologically friendly. So, understood that the availability of bioactive compounds is always insufficient to meet the needs of the final development test [14].

Marine invertebrates harbour a high abundance of symbiotic microorganisms that also synthesize secondary metabolites the same as their hosts [15-16]. Some evidence reported that marine invertebrate-associated bacteria are real producers of many bioactive compounds [17-18]. Hence, marine invertebrate-associated bacteria could be used as an alternative source to search for biologically active substances. Moreover, these bacteria are the most under-explored and only about 2% of pure cultures have been successfully cultured [19]. It is believed that there are still a few parts of unexplored cultivable gorgonian-associated bacteria that exist in these environments. Therefore, the research on gorgonian *Plexaura sp.* associated bacteria producing bioactive properties against multidrug-resistant *A. baumannii* is relevantly suitable to be conducted.

Materials and Methods

Gorgonian sampling.

Samples were collected at three-point locations around Arborek Island, Raja Ampat Regency, West Papua Indonesia in March 2020 by scuba diving. Three-point locations in the west, north, and east parts of the island, namely Arborek (00° 33' 55,81" LS 130° 30' 29,03" BT), Lalosi (00° 33' 51,37" LS 130° 29' 48,36" BT), and Batu Besar (00° 33' 32,05" LS 130° 31' 36,71" BT) were selected randomly for sampling (Figure 2). Gorgonians were sampled by scuba diving at 10-12 meters in depth. Gorgonian *Plexaura sp* were photographed, taken into a Ziploc, and put into the icebox. The gorgonian samples were identified based on their morphology by referring to Tuti and van Ofwegen's book on gorgonian identification [20].



Fig. 2. Arborek Island, Raja Ampat sampling sites (Google Map, 2020)

Bacterial isolation

The gorgonian samples were crushed with mortar and pestle, then made three dilutions. The 1 milliliter of dilution was dropped on Zobell Marine Agar (ZA) by the spread plate method and incubated at 27°C. The bacterial isolates to be purified were selected based on the different colony morphology. Purification was carried out on ZA media by streak method and incubated at 36°C.

Antibacterial assay

The agar plug method was used to assay the antimicrobial activity of bacterial isolates against nosocomial infection multidrug-resistant *A. baumannii*. All bacterial isolates were refreshed on ZA media and incubated for 3x24 hours at 36°C. After that, each bacterial isolate was cut into a circular shape and put on agar growing pathogen and incubated at 36°C. Bacterial isolates that have antibacterial activity will form an inhibition zone around their colonies.

PCR amplification of 16S rRNA gene

The partial 16S rRNA gene of selected Gorgonian-associated bacteria was amplified, purified, and sequenced according to *Radjasa* [19] methods.

Detection of PKS and NRPS gene clusters

Gene detection was done using specific primers consisting of PKS I (KS α -F and KS α -R) at 700 bp – 800 bp, PKS II (IIPF6 and IIPR6) at 600 bp – 650 bp, and NRPS (A2gamF and A3gamR) at 200 bp – 300 bp. Amplification was carried out using a PCR mix with the same composition as the DNA amplification in the previous step [21-22].

GenBank Accession Number Reference of selected isolates

The 16S rRNA sequence's *accession numbers* of isolates were deposited in GenBank including OK353798, OK426386, OK353799, OK426387, and ON860637 for the isolates RA17-2, RA17-9, RA-17-14, RA17-22, and RA17-23, respectively.

Results and discussion

Isolation and screening of bacterial isolates

The agar plug method was used to screen the twenty-nine gorgonian-associated bacterial isolates for their antibacterial activity. The results showed that 5 of the 29 (17.2%) isolates, RA17-2, RA17-9, RA17-14, RA17-22, and RA17-23 inhibited the growth of multidrug-resistant *A. baumannii*. These selected bacterial isolates were characterized morphologically and biochemically (Table 1 and Figure 3).

Table 1. Morphological characterization of isolates and pathogenic assay

Isolate	Morphological colony:					Pathogenic test	Inhibition Zone (mm)
	Colour	Size	Shape	Margin	Elevation		
RA17.1	white	medium	round	smooth	smooth	-	-
RA17.2	white	medium	round	spikey	smooth	+	2.00
RA17.3	white	medium	round	flat	smooth	-	-
RA17.4	yellow	medium	round	flat	smooth	-	-
RA17.5	white	medium	round	flat	smooth	-	-
RA17.6	white	medium	round	flat	smooth	-	-
RA17.7	white	medium	round	flat	smooth	-	-
RA17.8	white	medium	round	flat	smooth	-	-
RA17.9	white	small	round	flat	smooth	+	3.90
RA17.10	white	small	round	flat	smooth	-	-
RA17.11	yellow	medium	round	flat	smooth	-	-
RA17.12	yellow	medium	round	flat	smooth	-	-
RA17.13	white	medium	round	flat	smooth	-	-
RA17-14	white	medium	round	flat	smooth	+	1.40
RA17-15	yellow	small	round	flat	smooth	-	-
RA17-16	yellow	medium	round	flat	smooth	-	-
RA17-17	white	medium	round	flat	smooth	-	-
RA17-18	yellow	medium	round	flat	smooth	-	-
RA17-19	white	medium	round	flat	smooth	-	-
RA17-20	white	medium	round	flat	smooth	-	-
RA17-21	yellow	medium	round	flat	smooth	-	-
RA17-22	yellow	medium	round	flat	smooth	+	8.01
RA17-23	yellow	medium	round	flat	smooth	+	2.05
RA17-24	white	medium	round	flat	smooth	-	-
RA17-25	white	medium	round	flat	smooth	-	-
RA17-26	yellow	small	round	flat	smooth	-	-
RA17-27	white	medium	round	flat	smooth	-	-
RA17-28	yellow	medium	round	flat	smooth	-	-
RA17-29	white	medium	round	flat	smooth	-	-

A 16S rDNA gene and a phylogenetic analysis

The results demonstrated that RA17.2, RA17-9, RA17-14, RA17-22 and RA17-23 isolates were closely related to *Nitratireductor aquimarinus*, *Virgibacillus pantothenicus*, *Virgibacillus dokdonensis*, *Bacillus flexus*, and *Nitratireductor aquimarinus*, respectively (Table 2). The Firmicutes phyla including the genus *Bacillus*, *Virgibacillus*, and Proteobacteria phyla (genera *Nitratireductor*) appear to associate with the gorgonian *Plexaura* sp. (Fig. 4).

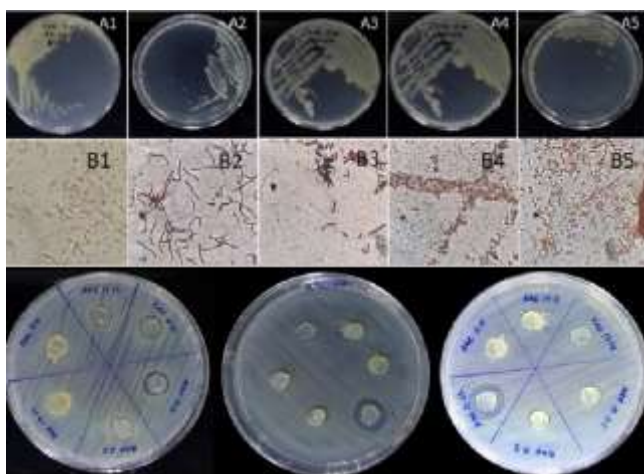


Fig. 3. Morphological characterization and a pathogenic assay of gorgonian bacterial isolates
(Note: A1-A5: culture of 5 selected isolates; B1-B5: microscopic photograph of selected isolates and antibacterial assay)

Table 2. Molecular identification of gorgonian-associated bacteria with antipathogenic property

Isolate	Closely related Blast	Acc.no.Reff.	Acc.no. isolate	Identity (%)
RA17-2	<i>Nitratireductor aquimarinus</i> strain NMX3	MT071320.1	OK353798	97.74
RA17-9	<i>Virgibacillus pantothenicus</i> strain OL03	JN791392.1	OK426386	100
RA17-14	<i>Virgibacillus dokdonensis</i> strain SMS-9-15	MK622389.1	OK353799	100
RA17-22	<i>Bacillus flexus</i> strain K3.2	MT299641.1	OK426387	100
RA17-23	<i>Nitratireductor aquimarinus</i> strain NMX3	MT071320.1	ON860637	100

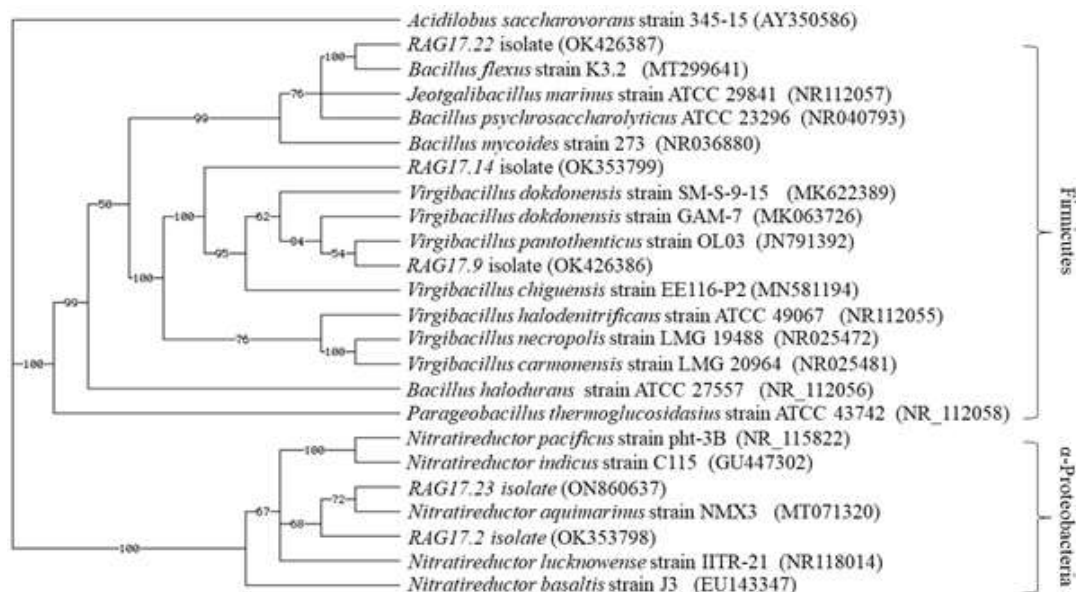


Fig. 4. Phylogenetic tree of *Pleaxaura*-associated bacteria with the antipathogenic property. *Acidilobus saccharovorans* strain 345-15 was used as an outgroup

PKS-NRPS gene clusters detection

The results of the amplification of PKS-I, PKS-II, and NRPS genes are visualized in figure 5. Based on the PCR results, one isolate of the PKS-I gene, RA17-2 strain, was detected. The other bands on PKS-II and NRPS appeared, however, these bands were neither PKS-II nor NRPS genes due to differences in the size of base-pair length.

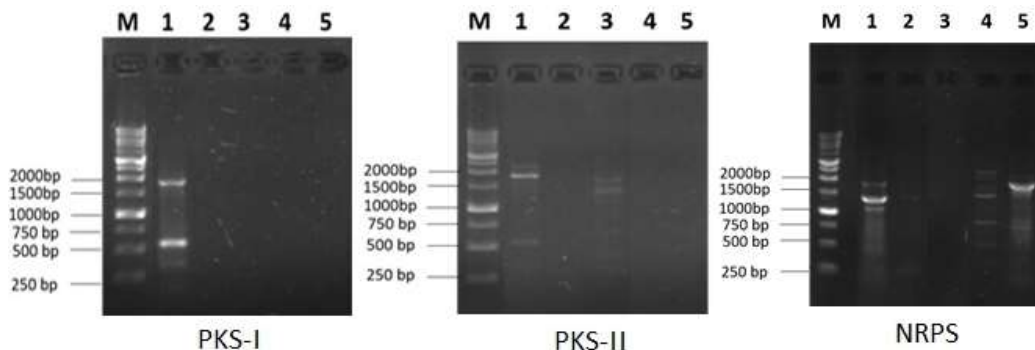


Fig. 5. NRPS, PKS-II, and PKS-I cluster genes of antipathogenic isolates
(Note: 1: RA17-2, 2: RA17-9, 3: RA17-14, 4: RA17-22 and 5: RA17-23)

Recently, both dependent and independent cultural techniques were used extensively to explore marine invertebrate-associated bacterial communities [23-24]. Although newly developed culture-independent molecular techniques have proved to be a promising tool to investigate population diversity and ecological significance, however, dependent cultural techniques are still useful for searching for bacterial potency [25-27]. By way of this study, 29 bacterial strains were isolated from gorgonian *Plexaura sp* of Arborek, Raja Ampat, West Papua. These isolates were screened, selected, and identified for their antibacterial activity through in vitro inhibitory activities/plug agar, polyphasic studies, and PKS-NRPS gene detection. The results showed that 5 of 29 bacterial isolates (17.24 %) could inhibit the growth of the *A. baumannii* bacterial test (Table 1; Figure 3). Several previous studies of gorgonian-associated bacteria with antibacterial activities had already been reported. Zhang [28] demonstrated that 72 of 123 (58.54 %) actinobacteria associated with gorgonian showed antibacterial activities against marine pathogenic bacteria. *Gnanambal* [29] also informed that 36 of 354 gorgonian *Subergorgia suberosa* and *Junceella juncea*-associated bacterial isolates demonstrated antibacterial activities against several human and fish pathogens. This evidence revealed that Gorgonian-associated bacteria serve as potential sources for searching for antibacterial compounds that are highly generous and various.

These 5 isolates were selected and identified molecularly and revealed as *Nitratireductor*, *Virgibacillus*, and *Bacillus* genera. Polyphasically, the strains RA17-2, RA17-9, RA17-14, RA17-22, and RA17-23 were closely related to *Nitratireductor aquimarinus*, *Virgibacillus pantothenicus*, *Virgibacillus dokdonensis*, *Bacillus flexus*, and *Nitratireductor aquimarinus*, respectively. The sequences of 16S rDNA of these isolates have been deposited in the NCBI and DDBJ under accession no. OK353798, OK426386, OK353799, OK426387, and ON860637 (Table 2; Figure 4). Among these bacterial genera, marine *Bacillus* strains are the most familiar for producing antibiotic compounds. Some articles regarding to *Bacillus* metabolites and their antimicrobial activities have been published [30-33]. Further, Tran [34] reported that more than thirteen hundred *Bacillus*, composed of 27 different species, are capable of producing antimicrobial activity. However, *Bacillus flexus* species reported in this study are not included in this composition. Hence, this bacterial species is an additional potential source of novel antibiotic compounds from the *Bacillus* genera. Other genera found in this study,

marine *Virgibacillus* sp. is also reported to have antibacterial properties against MDR *S. aureus*, *E. coli*, *Enterobacter* [35], *Vibrio parahaemolyticus* [36], and antioxidant [37]. Different from the genus *Bacillus* and *Virgibacillus*, no reports were found regarding *Nitratireductor* genera with antibacterial properties. To our knowledge, this might be the first report on polyphasic identification of antibiotic-producing bacteria associated with gorgonian *Plexaura* sp from Raja Ampat waters, West Papua, Indonesia. Antibacterial active compounds produced by the five selected strains in this study were not detected for the presence of PKS and NRPS gene clusters, except for PKS-I of the RA17-22 isolate (Figure 5).

These results indicated that both PKS and NRPS genes were not involved in the antipathogenic activity of RA17-2, RA17-9, RA17-14, and RA17-23 isolates. While the presence of the PKS-1 gene in the RA17-22 isolate might be responsible for antibacterial synthesis. On the other hand, Zhang [28] reported that both PKS and NRPS were involved in the antibacterial activity in *Lysobacter enzymogenes* OH11. This isolate was selected for further study in the discovery of novel antibiotic compounds due to showing promising results for the treatment of microbial infection.

Conclusions

The five gorgonian-associated bacterial isolates with antibacterial property which were identified as *Nitratireductor aquimarinus* strain RA17-2, *Virgibacillus pantothenicus* strain RA17-9, *Virgibacillus dokdonensis* strain RA17.14, *Bacillus flexus* strain RA17.22, and *Nitratireductor aquimarinus* strain RA-17.23 showed potential inhibitory activity against test bacteria on screening. The antibacterial activity test on the metabolite crude extract showed that all isolates had antibacterial activity against *A. baumannii*. These results indicate that gorgonian-associated bacteria isolated from pristine areas produce potential bioactive compounds and facilitate the discovery of new antibacterial agents.

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