Research Journal of Microbiology

ISSN 1816-4935

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Research Journal of Microbiology

ISSN 1816-4935
DOI: 10.3923/jm.2017.154.160

Research Article
Antipathogenic Activity of Bacteria Associated with Acroporid Corals Against Black Band Disease of Karimunjawa, Indonesia

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Abstract

Background and Objective: Black Band Disease (BBD) causing tissue loss and mortality has come out as a serious threat to coral reefs. This disease has been reported from several coastal regions in the world, however little is known the procedures for controlling infectious diseases of this corals. The purposes of this study were to screen and characterize of coral-associated bacteria having antipathogenic potency against BBD coral disease. **Materials and Methods:** One hundred and eighty three healthy coral derived bacterial isolates were screened. Thirty four strains were selected for their ability to inhibit BBD pathogenic isolates. The disc-diffusion method were used to rescreen for their antipathogenic property against BBD pathogenic isolates. Then, the 16S rDNA approach and microbiological characteristics were used to identify bacterial selected. **Results:** Thirty four (18.58%) strains showed their ability to inhibit the growth of BBD pathogenic isolates. The NM1.2, NM1.8 and NM1.9 isolates were selected according to their consistency of BBD inhibition. The DNA sequences analyses showed these isolates belonged to only one major groups of γ-proteobacteria. The NM1.2, NM1.8 and NM1.9 isolates were all closely related to genus *Pseudoalteromonas*. **Conclusion:** Even though the low diversity of bacterial phylotypes were present within the coral family Acroporidae, however, this study provides the first evidence of acroporid coral bacteria possessing antipathogenic activity against BBD coral disease that can be recovered from corals. The sequence data of these antipathogenic strains, *Pseudoalteromonas flavipulchra* strain NM1.2, *Pseudoalteromonas maricaloris* strain NM1.8 and *Pseudoalteromonas piscicida* strain NM1.9, in this study have been deposited to the GenBank data library under the accession number LC184593, LC184594 and LC184595.

Key words: Coral disease, Acroporidae, black band disease, antipathogen, *Pseudoalteromonas*

Received: October 02, 2016 Accepted: January 19, 2017 Published: March 15, 2017

Citation: A. Sabdono, D.P. Wijayanti and Sarjito, 2017. Antipathogenic activity of bacteria associated with acroporid corals against black band disease of Karimunjawa, Indonesia. Res. J. Microbiol., 12: 154-160.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Karimunjawa archipelago, which forms a chain of 27 islands, represents several ecosystem types such as coral reefs, mangroves and seagrass. This archipelago has been considered to be one of seven all Marine National Park in Indonesia since 2005 with more than 50 genera of corals[1]. Four among six extant hermatypic coral genera that include in Acroporidae family, *Acropora* sp., *Montipora* sp., *Anacropora* sp. and *Astreopora* sp., appear in Karimunjawa. This family exist in colonial, hermatypic that have all growth forms with small corallites and poorly developed columellae[2]. Acroporid coral was chosen as an object in the study because of the continues outbreak of BBD in this family that leads to degrading of coral ecology in Karimunjawa[3].

During the last few years, Black Band Disease (BBD) has spread worldwide and caused large-scale mortalities and severe damage to coral reefs[4-6]. The BBD was first reported in 1973 in the Caribean[7]. Three decades after, BBD was first reported at Karimunjawa archipelago[8] and has remained widespread since. This disease then has been observed throughout the Indonesia islands, affecting primarily the genera *Montipora* sp., *Acropora* sp., *Diploastrea* sp. and *Pavona* sp.[9-11,3]. The syndrome is characterized by a darkened areas, band-like microbial mat in the coral tissues at the interface between healthy coral tissue and exposed skeleton (Fig. 1). The BBD mat that composes the band is dominated by *Phormidium corallyticum*, genus *Oscillatoria*, sulfate-reducing bacteria *Desulfovibrio* spp. and sulfide oxidizing bacteria *Beggiatoa* spp[6]. However, recent studies utilized molecular techniques showed that newly bacterial strain *Roseofilum reptotaenium* presents in BBD mats[12,13]. The band moves across coral colonies that degrading, killing coral tissue, causing necrosis and colonizing by algae[14,15]. Progression rates of BBD lesions differ among host species, geographics and seasons. Sutherland *et al.*[6] reported the progression rate of BBD in coral Carribean was 3 cm day^{-1}, while Sato *et al.*[16] reported the progression rate of BBD in 3.2-5.2 cm day^{-1}. Four years after, Aeby *et al.*[17] reported that the BBD pathogenic bacteria were more virulent with average rate of tissue loss of 5.7 cm^2 day^{-1} over a 2 months period. Furthermore, Chen *et al.*[18] and Sato *et al.*[19] formulated the model to predict the dynamics of BBD development under environmental drivers from healthy coral colonies transitioning into a cyanobacterial patches and transitions into BBD. Garcia *et al.*[20] reported coral metabolic status in health and disease that suffering from the increase in global and local threatening events.

The use of bacteria for the treatment of insect pest, weed and plant disease has had a long history[21]. Recently, antagonistic bacteria were used for biological control of pathogens infecting plant roots[22]. Numerous studies have demonstrated the ability of several antagonistic bacteria to suppress diseases caused by pathogens[23]. However, there are limited effort for preventing or treating infectious diseases of corals. Efrony *et al.*[24] used bacterial viruses (bacteriophages) to control the spread of White Plaque (WP) coral diseases. While, Raymundo *et al.*[25] attempted to stop the migration of BBD cyanobacterial mats from active lesions by covering the lesion with epoxy. However, none of them made any success on their efforts. The excellent approach is urgently needed to handle the spread of coral diseases and biological control might be the appropriate choice. The objectives of this study were to screen and identify antipathogenic BBD of bacteria associated with acroporid corals.

MATERIALS AND METHODS

Coral sampling and bacterial isolation: Acroporid corals, *Acropora* sp. and *Montipora* sp. are found on open reef bottoms of around Genting waters. Specimens of those hard corals were collected by scuba diving at depths of 5-6 m of the

Fig. 1: Black band disease on acroporid coral genus *Montipora* sp.

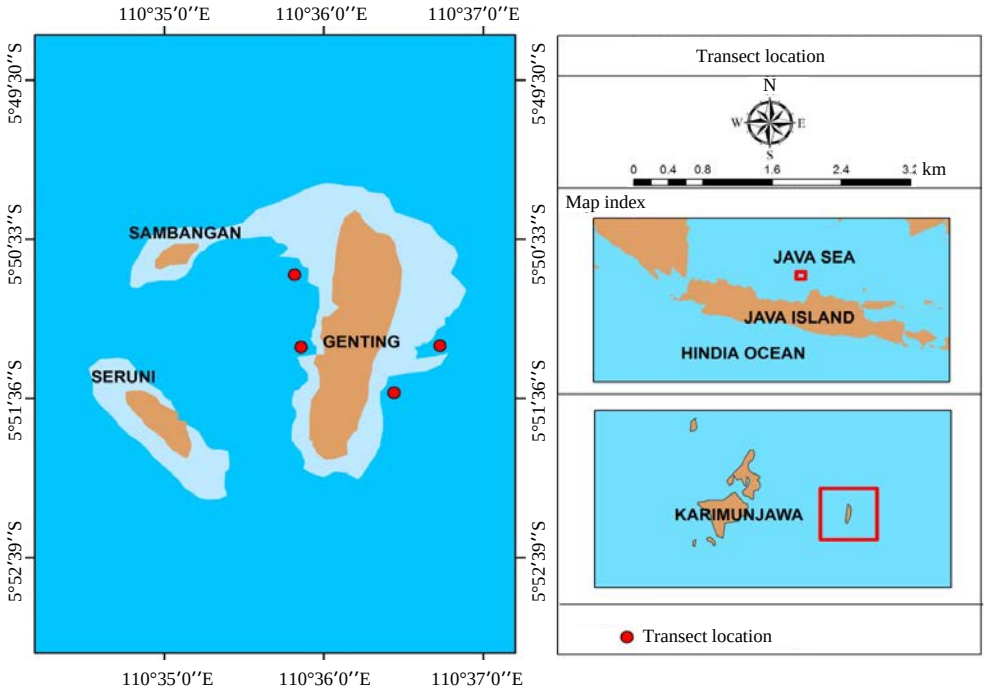

Fig. 2: Sampling site locations

Genting island on March, 2015 (05°50′44.3″ LS 110°35′0.07″ BT). Figure 2 shows the sampling site location. Individual specimens were placed separately into plastic bags to avoid contact with air and brought to the surface. The samples were kept individually in plastic bags containing natural seawater until processing within a few hours after collection. Bacterial isolation was conducted similar to previously study with slightly modification[26]. Coral fragments were rinsed with sterile seawater and scraped off with a sterile knife. The resultant tissues were serially diluted, spread on 1/2 strength ZoBell 2216E marine agar medium and incubated at room temperature for for 2 days. Based on of morphological characteristics, bacterial colonies were randomly selected and purified by streak method.

Antipathogenic assay: Acroporid bacterial symbionts were assayed for antipathogenic activity by overlay and agar diffusion methods as previously described[27]. Each BBD strains were designed for antipathogenic effect study. The ability of coral associated bacteria to inhibit the growth of BBD bacteria was performed by using an overlay test method. Aliquots culture of each BBD bacterium in the logarithmic growth phase (ca. 10^8 cells mL^{-1}) was mixed with Zobell 2216E soft agar medium, which were then poured on to the respective agar surface, previously inoculated with coral associated bacteria and incubated for 96 h. The plates were then incubated at room temperature for 48 h. Antipathogenic activity was defined by the formation of inhibition zones around the bacterial colonies.

Nine coral bacteria selected from overlay test were reperformed by using the agar disc-diffusion method. Hundred microliters of each BBD isolate culture in the logarithmic phase (ca. 10^9 cells mL^{-1}) was spread on agar medium. Paper disks (Φ 8 mm; Advantec, Toyo Roshi, Ltd., Japan) containing 35 µL of selected strain was placed on the respective agar surface. The plates were then incubated at room temperature for 48 h. Antipathogenic activity was defined by the formation of inhibition zones greater than 9 mm around the paper disk.

Microbiological characterizations: Coral selected bacteria were grown in Zobell 2216E medium and underwent further microscopic and biochemical evaluations. Photomicrograph was used to determine the morphology of the isolates. While standard gram staining, motility and biochemical characterizations based on Bergey's manual of determinative bacteriology were used to determine their biochemical properties.

Molecular taxonomy identification: The protocols of molecular identification were conducted according to previously a method described[27]. The DNA antipathogenic isolate for PCR analysis was gained from bacterial cells and extracted by using freeze and thaw method. Primers [(forward primer 8-27: 5′-AGAGTTTGATCCTGGCTCAG-3′[28] and reverse

primer 1510-1492: 5'-GGTTACCTTGTTACGACTT-3'[29]] were used to amplify 16S rDNA. The resulting 16S rDNA sequences corresponding to the genotype were analyzed for homologies with sequences in the data base using BLAST searching. CLUSTAL X was used for multiple alignment/pairwise the DNA sequence[30]. Phylogenetic analysis was performed with the Phylogenetic Analysis Using Parsimony (PAUP version 4) software package[31].

Nucleotide sequence accession numbers: Nucleotide sequences of the 16S rDNA from three antipathogenic BBD isolates obtained in this study have been deposited in the GenBank database under accession number LC184593, LC184594 and LC184595. The accession numbers of 16S rDNA of other strain cited and used as comparison.

RESULTS AND DISCUSSION

Recently, the number of coral diseases and the geographic distribution of diseases have reported increasingly. In concurrent, several studies have also reported the antimicrobial activity produced from scleractinian corals. Marquis *et al.*[32] reported that several scleractinian species have antipathogenic activities. However, it is very few studies have been conducted to understand the role of coral associated bacteria in maintaining coral health[33]. In this study recorded that approximately 19% (34 isolates) healthy coral bacteria associated with acroporid corals demonstrated antipathogenic activity (Fig. 3). Similar result was reported by Ritchie[34] that nearly 19% of the cultured bacteria isolated from the *Acropora palmata* showed antipathogenic activity against white pox coral disease. Shnit-Orland and Kushmaro[35] showed that more than 20% (21) strains isolated from scleractinian corals demonstrated antipathogenic activity. While, Nissimov *et al.*[36] found that only 9 (6%) strains isolated from healthy coral *Oculina pathagonica* showed antipathogenic activity against the causative agents *Vibrio shiloi*.

Based on the result of overlay tested, 9 isolates that capable of inhibiting more than 1 BBD isolate were re-examined by using agar disc-diffusion method to confirm the previous observation (Fig. 4). The experiment showed that NM1.8 and NM1.9, NM1.2 and TF1 isolates could inhibit the growth of 4, 4, 4 and 2 pathogenic BBD strains, respectively. While 5 other isolates could inhibit only 1 BBD isolate (Table 1). Inhibition zones that formed both on the overlay and the agar diffused methods indicated the possible production of cell associated antipathogenic BBD activity. This should be investigated further experiments to determine the nature of this interaction. It could be possible that coral mucus and its

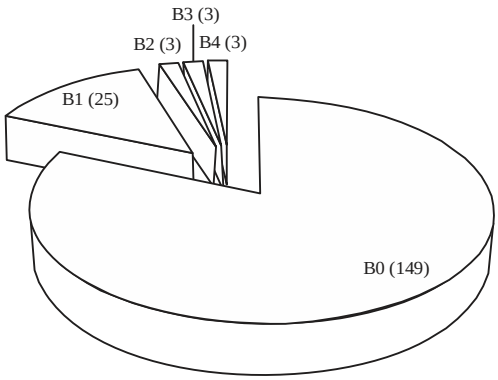

Fig. 3: Proportions of antipathogenic bacterial communities against the BBD, B0: No inhibition, B1: Inhibit 1 BBD, B2: Inhibit 2 BBD, B3: Inhibit 3 BBD, B4: Inhibit 4 BBD isolates

Fig. 4: Antipathogenic assay on disc-diffusion agar method

Table 1: Mean and Standard Deviation (Mean±SD) of antipathogenic assay by using diffusion agar

		Agar diffusion (mm)			
Isolate code	Overlay	BBD1	BBD2	BBD3	BBD4
NM1.2	++++	4.56±0.08	9.91±0.36	7.76±0.41	9.22±0.22
NM1.8	++++	5.81±0.10	9.54±0.20	4.71±0.32	7.95±0.62
NM1.9	++++	8.71±0.42	10.96±0.04	10.19±0.16	9.96±0.15
TF1	+++	-	-	2.17±0.02	4.89±0.25
TAF4.5	+++	-	-	0.43±0.54	-
NM1.5	+++	-	-	10.89±0.09	-
KPSH5	++	-	-	2.62±0.11	-
GASH2.4	++	-	-	3.67±0.19	-
TPSH1.1	++	-	-	-	3.31±0.06

+: Inhibit 1 BBD isolate, ++: Inhibit 2 BBD isolates, +++: Inhibit 3 BBD isolates, ++++: Inhibit 4 BBD isolates

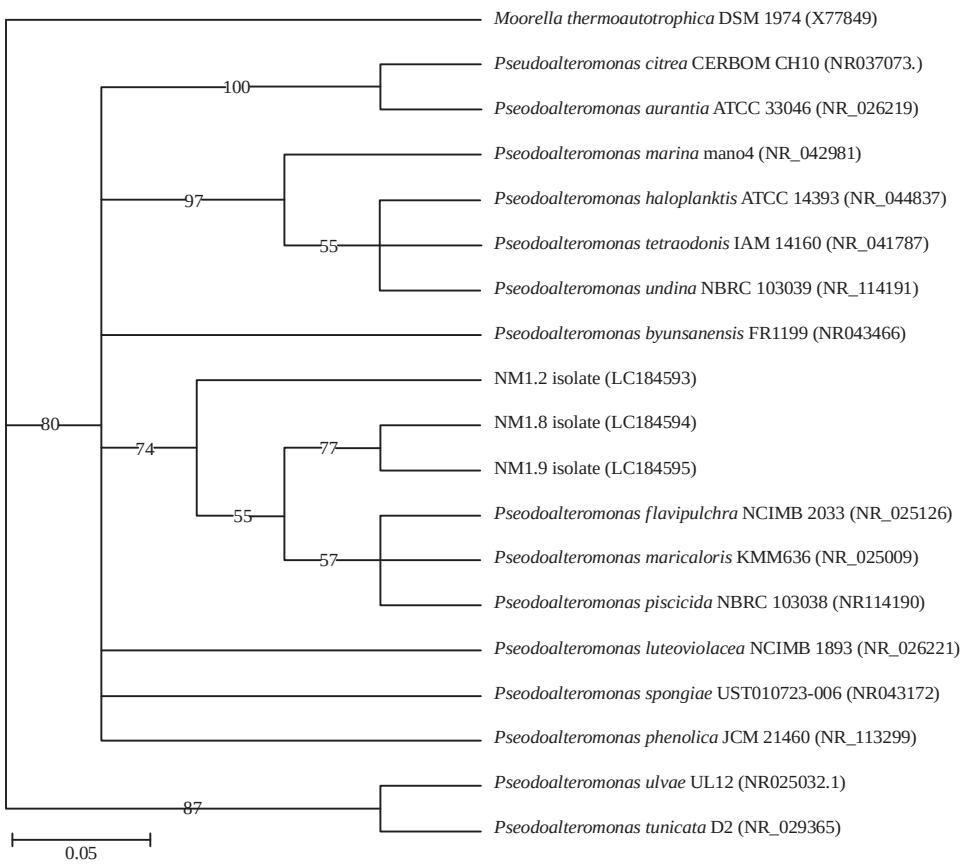

Fig. 5: Phylogenetic tree based on the 16S ribosomal DNA sequence data showing the relationships of representative strains with the most closely related bacteria identified in the GenBank database, *M. thermoautotropica* was used as out group

Table 2: Characterization of representative BBD-antipathogenic coral bacteria

Isolates	Bacterial groups	Closest relative in GenBank by BLAST	Accession No.	Homology (%)
NM1.2	γ-Proteobacteria	*Pseudoalteromonas flavipulchra* NCIMB 2033	NR_025126	98
NM1.8	γ-Proteobacteria	*Pseudoalteromonas maricaloris* strain KMM636	NR_043172	98
NM1.9	γ-Proteobacteria	*Pseudoalteromonas piscisida* strain NBRC 103038	NR_114190	98

associated microorganisms play an important role in promoting beneficial microbial communities and the protection of the coral[28,29].

The NM1.2, NM1.8 and NM1.9 isolates that could inhibit the growth of 4 pathogenic BBD strains were subjected to 16S rDNA sequence analysis. The sequencing results were analyzed to their homology to the sequences of known bacteria in the GenBank. The all sequences of the isolates have 98% similarity to the bacterial sequence in the GenBank (Table 2). All of these antipathogenic coral bacteria were belonged to γ-Proteobacteria group. Zhang *et al.*[37] reported that the γ-Proteobacteria was the major bacterial group in scleractinian corals. In addition, Ibrahim[38] reported that bacterial community of gorgonian coral *Eunicea fusca* were composed mainly of the bacterial group α-Proteobacteria and γ-Proteobacteria. The BLAST analysis showed that NM1.2, NM1.8 and NM1.9 strains were closely relative to *Pseudoalteromonas flavipulchra* strain NCIMB 2033, *Pseudoalteromonas maricaloris* strain KMM636 and *Pseudoalteromonas piscicida* strain NBRC 103038, respectively. In the previous study have shown similar genus *Pseudoalteromonas* to be present in healthy coral *O. patagonica* with antipathogenic property[36]. The 16S rRNA gene sequences of 3 bacterial selected isolates were subsequently aligned to construct a phylogenetic tree (Fig. 5). Molecular phylogenetic identification of strains revealed low diversity of cultivable bacteria which belonged to one genus only. Microbiological characterization demonstrated that NM1.2, NM1.8 and NM1.9 strains isolated from the tissues of acroporid corals were Gram-negative, aerobic bacteria and decomposed starch. The cells of these bacteria were rod-shaped with a single polar flagellum and they formed

Table 3: Microbiological characterization of 3 antipathogenic BBD isolates

	Isolates		
Characters	NM1.2	NM1.8	NM1.9
Gram	-	-	-
Shape	Rod	Rod	Rod
Spora	+	+	+
Motility	+	+	+
Aerobic	+	+	+
An aerobic	-	+[F]	+[F]
Catalase	+	+	+
Oxidase	-	+	+
Glucose acid	+	+	+
Carbohydrate (OF)	NC	NC	NC
Nitrate reduced	+	+	-
Gas from glucose	-	-	-
Indol	-	-	-
ONPG	-	-	+
VP	-	-	-
Hydrolysis			
Starch	+	-	+
Urea	-	-	+
Casein	+	-	+
Acid from AAS medium			
L-arabinose	-	-	-
Salicin	-	-	-
Sucrose	-	-	-
Xylose	-	-	-
Celibiose	-	-	-
Galactose	-	-	-
Rafinose	-	-	-
Gelatinase	v	v	v
Ulitization of nitrat	-	-	-

+: Reaction, -: No reaction, NC: No change, v: No assayed, F: Facultative

endospore (Table 3). These characters were corresponds to the results of the molecular taxonomic identification. The comparison of taxonomic identification of these bacteria on their microbiological properties indicated that they were highly similar.

CONCLUSION

In conclusion, the diverse acroporid corals-derived bacteria from the Genting islands, Karimunjawa have a limited metabolites with various antipathogenic activities. The presence of γ-Proteobacteria may play significant roles in antipathogenic BBD communities. These bacterial group can be of potential use to biocontrol agents of BBD coral disease.

SIGNIFICANT STATEMENTS

- One hundred and eighty three bacteria of acroporid coral tissues was isolated, 19% of them could inhibit the growth of BBD pathogen
- Three isolates identified as genus *Pseudoalteromonas* sp., presented consistent and strong antipathogenic BBD activity
- These strains are useful as biocontrol agents, active against BBD coral pathogenic bacteria

ACKNOWLEDGEMENT

The author is very grateful to Mr. Handung Nuryadi and Sakti Muchlisin as assistant researchers at Integrated Laboratory of Diponegoro Semarang. This research was supported by Penelitian Unggulan Perguruan Tinggi (PUPT): No. 022/SP2H/LT/ DRPM/II/2016 tanggal 17 Pebruari 2016.

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