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Bacterial symbionts of acroporid corals: Antipathogenic potency against Black Band Disease

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Abstract. *Wijayanti DP, Sabdono A, Widyananto PA, Dirgantara D, Hidaka M. 2018. Bacterial symbionts of acroporid corals: Antipathogenic potency against Black Band Disease. Biodiversitas 19: 1236-1242.* Black Band Disease (BBD), an infectious coral disease which can cause a rapid decline of coral reefs, has appeared as a serious threat -to many reefs around the world including Karimunjawa National Park, Java Sea. Although it had been studied for more than 30 years, control of disease remains obscure. In the present research coral symbiont bacteria having antipathogenic activity against Black Band bacterial associate were screened and characterized. Fourteen out of 87 bacteria isolates derived from healthy corals showed antagonism against the Black Band bacterial strains. The isolates were then re-examined using disc-diffusion method to confirm the initial observation. The CI6 showed the strongest ability to inhibit BAFBB5, a bacterial strain associated with the BBD. Following the partial sequencings of 16S rDNA, the results indicated that the CI6 isolate was closely related to *Virgibacillus salarius* strain SA-Vb1, while the BBD associate isolate has strong relation with *Virgibacillus marismortui.* The results have shown that bacterial strains derived from healthy acroporid corals have potential to be used as a biocontrol agent against BBD.

Keywords: *Acropora*, antipathogenic activity, Black Band Disease, Karimunjawa, *Virgibacillus*

INTRODUCTION

Coral disease outbreaks are one of the main factors contributing to the decline of coral reefs globally (Weil et al. 2006). Diseases have increased in some occurrence and severity, number of coral hosts infected and the geographical extent of outbreaks (Harvell et al. 2004). Increased disease prevalence also may be related with in-balance in the coral essential microbial community that affects the relationship between coral host and zooxanthellae to form coral holobiont to fight invasion by other pathogenic microbes (Bourne et al. 2009).

Black Band Disease (BBD) is known as one of most destructive disease which caused large-scale mortalities and severe damage to coral reefs in the world (Dinsdale 2000; Al-Moghrabi 2001; Sutherland et al. 2004; Weil 2004; Yang et al. 2014). BBD was described as darkly pigmented microbial mat that necrotizing the healthy coral tissue which leads to mortality of the colonies (Beeden et al. 2008). Currently, various strain of cyanobacteria and diverse bacteria community was identified as the precursor of the BBD (Gantar et al. 2011; Buerger et al. 2016). Various environmental factors were attributed to the onset and progression of the disease. Light was thought to be involved in the pathogenesis of BBD. Mat of filamentous bacteria will be down when light levels are high (Viehman and Richardson 2000). Nutrient enrichment was reported to increase the progression of BBD (Voss and Richardson 2006). However, various progression rates of BBD mats were reported among different host, geographic range and season. Sutherland et al. (2004) reported that the progression rate of BBD lesion in the Caribbean coral was 3 cm d^{-1}. Different progression rates were observed when combination factors of light and temperature were used in a controlled experiment. The greatest progression rate was observed in the highest temperature and light treatment (5.2 mm d^{-1}) (Sato et al. 2011). While a peculiar slow progression was reported from Indian Ocean (Montano et al. 2015). Elevated temperature was thought contributed to the incidence of the disease (Richardson and Kuta 2003).

To date, no major disease outbreaks have yet been reported from Indonesia. Little is known about coral disease and its status in Indonesia, despite large area of the coral reefs (Johan et al. 2016). Black Band Disease (BBD) is the most notable disease reported from Indonesian waters. Prevalence and incidence of BBD were reported from Seribu Island with Montipora is the main genus infected by the disease (Johan et al. 2012; Delpopi et al. 2015; Johan et al. 2016). BBD was also reported from Lembata, East Nusa Tenggara (Abrar et al. 2012); and Wakatobi National Park (WNP) (Haapkylä et al. 2007; Muller et al. 2012) with *Montipora* sp. (Abrar et al. 2012; Johan et al. 2012; Johan et al. 2016) and *Diploastrea heliopora* (Muller et al. 2012) are the main coral suffered from the disease. *Myroides odoratimimus* strain BBD1, *Bacillus algicola* strain BBD2 and Marine Alcaligenaceae bacterium strain BBD3 were reported as causative agents of the disease at Karimunjawa (Sabdono and Radjasa 2006a).

Various strategies are attempted to stop the spreading of disease. It is common to control the spreading of the disease using other microorganisms especially in plants (Shoda, 2000). In coral, antagonistic potential activity of *Vibrionales* and *Alteromonadales* isolated from healthy coral *Montastrea annularis* was observed against the growth of the coral pathogen *Vibrio* at 25ºC (Rypien et al. 2009). Efrony et al. (2007) used lytic bacteriophages to control the growth of *Thalosomonas loyaeana,* causative agent of White Plague disease in *Favia favus.* Antibacterial activity developed from coral-associated bacteria were used to inhibit the growth of BBD causative agents (Gantar et al. 2011; Sabdono et al. 2014; Sabdono et al. 2017). However, the emergence of new agents of BBD, prompted attempts to find more antibacterial activity to control the progression of BBD.

Occurrence of Acroporid BBD that leads to degrading of coral ecosystem in Karimunjawa Islands continued to observed in various researches (Sabdono et al. 2014; Sabdono et al. 2017). High tourism activities were thought triggered reefs deterioration (Taruc 2011). Increasing number of boats use for tourist leads to a decrease in water quality, while irresponsible tourist contributes to increase waste pollution (BTNKJ 2016). The present research was conducted to find the causative agent of BBD in the Acroporidae and the potency of healthy coral associate-bacteria that have antibacterial activity against the BBD causative agents.

MATERIALS AND METHODS

Study area

Karimunjawa was designated as a restricted area in 1986 based on the Decree of the Minister of Forestry (PHKA No. 123/ Kpts-II/1986) as an effort to protect biodiversity of the reefs from destructive fishing activities. It was subsequently designated as a formal Marine Protected Area based on Minister of Forestry Decree No. 74/Kpts-II/2001 (Campbell et al. 2013).

Apparently healthy and infected coral colonies were collected by scuba diving at depths of 5 to 6 m of sampling area on September 2015 (Figure 1). The coral tissues were removed using hammer and chisel by partially reclining coral colonies to cut off part of the tissue (crapping) that affected by the disease and healthy part. Fragments were kept in labeled zip lock plastic to avoid contact with open air before brought to the sea surface (Sabdono and Radjasa 2006a). Samples were processed soon after collection. Number of healthy coral colonies that were collected from each site and number of isolates obtained from healthy corals was shown in Table 1.

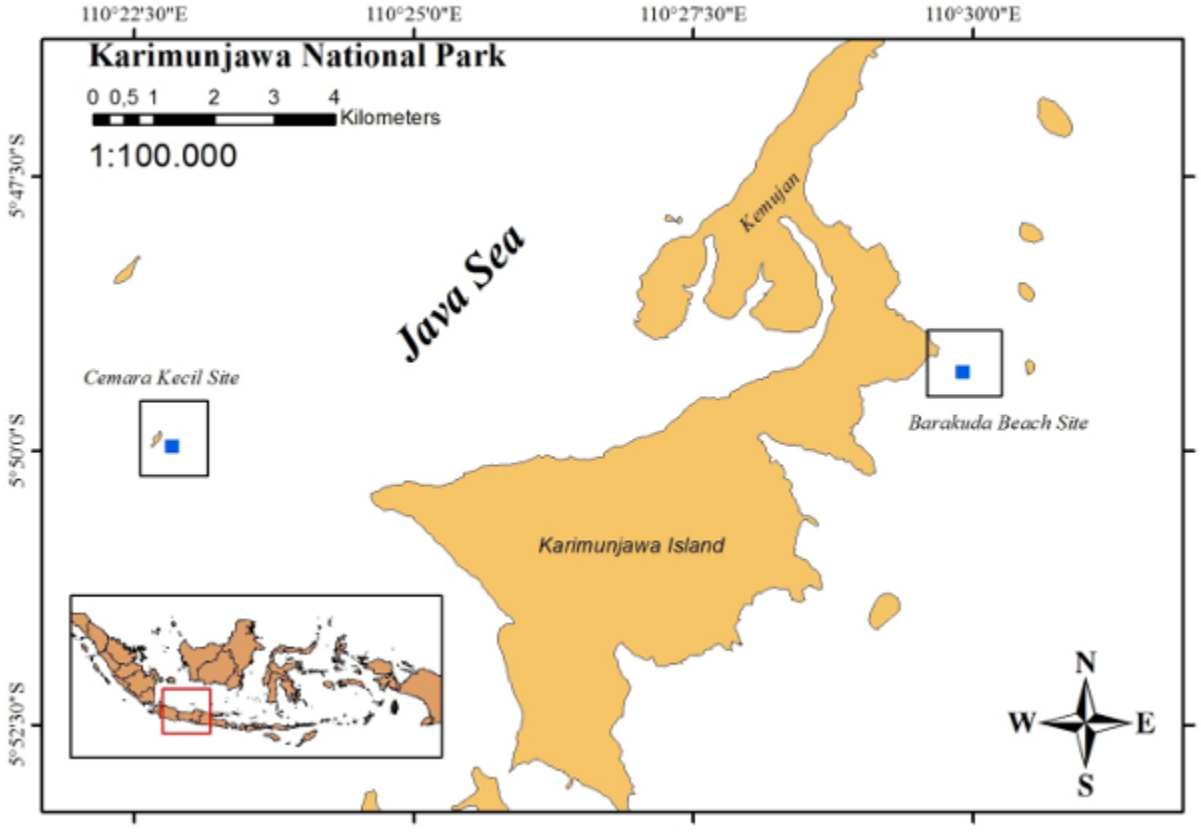

Figure 1. Samples were collected from Barakuda Beach (E 110°29'54,8" S 05°49'16,9") and Pantai Batu Putih (E 110°29'30,1" S 05°48'21,1") of Kemujan Island and Site I (E 110°22'50,5" S 05°49'57,7") and Site II (E 110°22'44,4" S 05°50'05,4") of Cemara Kecil Island, Karimunjawa Islands, Central Java, Indonesia

Table 1. Sampling location, name of healthy colonies collected and number of isolates obtained from healthy coral in Karimunjawa Islands, Central Java, Indonesia

Kemujan Island				**Cemara Kecil Island**	
Barakuda Beach (E 110°29"54.8" S 05°49'16.9")		Pantai Batu Putih (E 110°29'30.1" S 05°48'21.1")		(Site I) E 110°22'50,5" S 05°49'57,7" (Site II) E 110°22'44,4" S 05°50'05,4"	
Healthy coral samples (isolate code)	Number of isolates collected	Healthy coral samples (isolate code)	Number of isolates collected	Healthy coral samples (isolate code)	Number of isolates collected
Acropora muricata (BAF)	7	*Acropora hyacinthus* (PAH)	6	*Cyphastrea* sp. (CC)	6
Porites sp. (BP)	10	*Montipora* sp. (PM)	5	*Isopora palifera* (CI)	7
Favia sp. (BF)	7			*Acropora aspera* (CAA)	9
Montipora sp. (BMT)	12			*Acropora gomezii* (CAG)	6
				Acropora muricata (CAF)	6
				Stylophora pistillata (CSP)	6
Total isolates collected	47		11		40

Bacterial isolation was conducted followed the method of Sabdono and Radjasa (2006b) with minor modification. Scraped off coral tissues were then minced using mortar and pestle. The paste tissues were serially diluted, grown on a half strength ZoBell 2216E marine agar medium and incubated at room temperature for 2 days. Based on bacterial morphology, the colonies were then selected randomly and purified by streak method.

Antipathogenic assay

Antagonistic activity of the bacterial colonies was then observed by agar overlay and diffusion methods following description in Radjasa et al. (2007). Antipathogenic assay was done on each BBD bacterial strains. The ability of coral bacterial symbiont against BBD bacterial isolate was tested using an overlay test method. Aliquots culture in the logarithmic growth phase (ca 10^8 cells ml^{-1}) of BBD associated bacteria were mixed with Zobell 2216E soft agar medium. The soft agar- isolates mixed was then poured on to an agar medium surface that already contained coral bacterial symbiont and was incubated for 96 hours. The plates were then incubated for 48 hours at room temperature. Antipathogenic activity was observed when coral bacterial colonies demonstrated a halo formation to inhibit the growth of the BBD bacterial strain.

Interactions that demonstrated antagonism were rescreened for further investigation by using diffusion agar method. The culture of 100 µl of BBD associated bacteria in the logarithmic phase (ca. 10^9 cells ml^{-1}) was spread on to agar medium. To rescreened the BBD associated bacteria, filter paper discs (8 mm in diameter, Advantec Toyo Roshi Ltd, Japan), carrying 35 µl of selected coral bacterial isolates, were placed on the surface of medium. The plates were then incubated for 48 hours at room temperature. The inhibitory interaction was determined by the appearance of inhibition zones around the paper disc and was measured quantitatively.

Molecular taxonomy identification

Molecular identification of BBD associated bacteria and coral symbiont bacteria were conducted following a method described by (Radjasa et al. 2007). PCR product of antipathogenic DNA was obtained from selected coral symbiont bacterial cells that were extracted using freeze and thaw method. PCR amplification to amplify 16S rDNA was conducted using forward primer [(8-27: 5'-AGAGTTTGATCCTGGCTCAG-3' (Weisburg et al. 1991) and reverse primer 1510-1492: 5'-GGTTACCTTGTTACGACTT-3' (Reysenbach et al. 1992)]. BLAST searching was used to analyze homologies between the sequences obtained with bacterial sequences deposited in the database. Phylogenetic and multiple alignments/pairwise the DNA sequence analysis was performed with the MEGA 5.0 software package (Kumar et al. 2004).

Nucleotide sequences of the 16S rDNA from the antipathogenic BBD bacteria and the possible causative agent of the disease were already deposited to the GeneBank database under accession number LC260001 and LC259996.

RESULTS AND DISCUSSION

BBD was observed to attacked *Acropora muricata* at Pasir Putih Beach, Kemojan Island. The coral showed characteristic of BBD lesions since darkly pigmented microbial mat appeared formed a concentric necrosis band-like that revealed bare skeleton at the adjacent of the microbial mat (Beeden et al. 2008). Total 87 bacterial isolates were obtained from various collected healthy corals, while 8 strains were isolated from BBD bacterial mat of *A. muricata* (Figure 2)

Antipathogenic assay

Based on the overlay test, 14 isolates out of 87 (approximately 16%) bacteria isolates of healthy corals showed antagonism activity against BBD isolates. One isolate showed antagonism activity against two isolates of Black Band disease while 13 isolates only able to inhibited 1 isolate of BBD. The 14 isolates that managed to inhibit at least 1 BBD isolates were then reexamined by using agar disc-diffusion method to confirm previous observation.

Only 3 isolates successfully inhibited the growth of BBD isolates after the test. Strongest activity was shown by CI 6 isolate that could inhibit the growth of one BBD isolate BAFBB 5 by inhibition zone 3,66 ± 0,89 mm followed by PAH 6 isolate against BAFBB 3 with inhibition zone 1,93 ± 0,35 (mm) and CI 1 against BAFBB 5 with inhibition zone 1,76 ± 0,37 (mm) (Table 2).

The strongest isolate that showed capability to inhibit the growth of BBD bacterial strains, as well as the opponent strain, were then subjected to 16S rDNA sequence analysis. The sequencing results were analyzed to homology of deposited sequenced of known bacteria in the GenBank. The homology showed that the sequences have 99% similarity to the known bacterial sequence in the GenBank (Table 3).

Result of BLAST searching found that the healthy coral isolate CI 6 has strong relationship with *Virgibacillus salarius*. While BLAST searching of disease agent found that the BBD BAFBB 5 isolate showed similarity with *Virgibacillus marismortui* The accession numbers of 16S rDNA of other strain cited and used as well as comparison are presented in Figure 3.

Discussion

Coral disease outbreaks are now recognized as the main factors contributing to the global decline of coral reefs (Weil et al. 2006). Together with coral bleaching, coral disease risk more than 32% of reef-building corals around the world to face an extinction (Carpenter et al. 2008). Lesser et al. (2007) suggested that environmental changing contribute to coral's holobiont balance composition which triggered the appearance of pathogenic bacteria.

In this study, BBD was reported to be found at Karimunjawa Islands. BBD was observed at Pasir Putih Beach, Kemojan Island. BBD was also found at different island of Karimunjawa Islands (Sabdono and Radjasa 2006a,b; Hasbullah et al. 2012; Sabdono et al. 2017). Karimunjawa Islands currently is emerging as new tourist destination (Taruc 2011). Increasing tourist activities are thought contribute to the decrease of water quality (BTNKJ 2016). Daily dumping of sewage and other pollutants were thought as precursor of BBD incidence (Frias-Lopez et al. 2002). However, recent report from Seribu Island suggested no linear relationship between distance of BBD incidence from major population centers as most probable resource of sewage dumping and other pollutants (Johan et al. 2016).

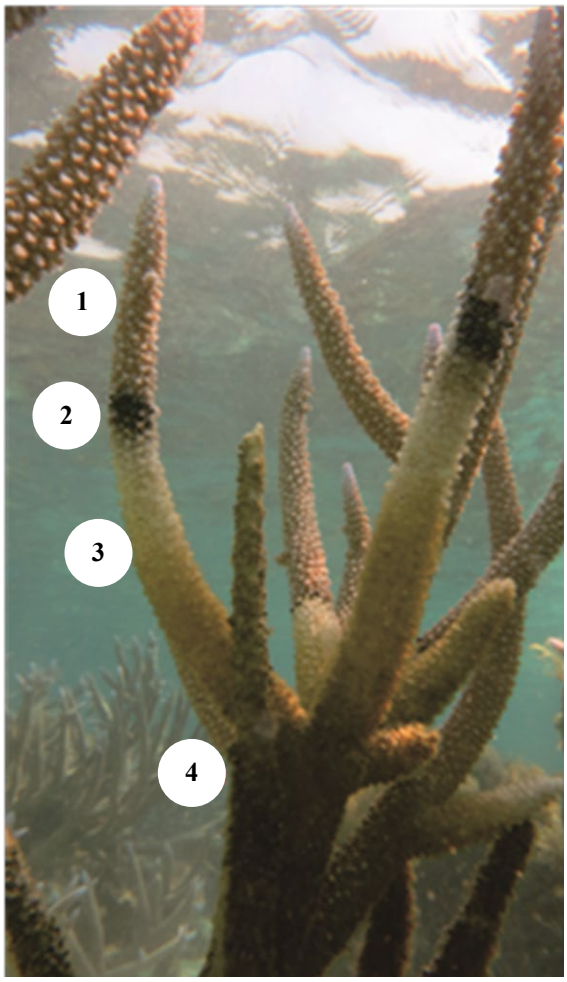

Figure 2. *Acropora muricata* infected by Black Band Disease in Kemujan Island, Karimunjawa National Park, (1) Alive tissue of the coral, (2) Lession of coral disease with Black Band mat, (3) Death area of the coral, (4) death area that has been overgrown by algae

Table 2. Mean and standard deviation (M± SD) of anti-pathogenic assay by using diffusion agar

Healthy coral isolates code	Pathogenic isolates code	Agar diffusion (mm)
CI 6	BAFBB 5	3.66 ± 0.89
PAH 6	BAFBB 3	1.93 ± 0.35
CI 1	BAFBB 5	1.76 ± 0.37

Tabel 3. BLAST analysis at GenBank; CI 6, the antipathogenic isolate derived from healthy coral; BAFBB 5, BBD causative agents

Isolate code	Length of nucleotide (bp)	Closest relationship	Homology (%)	Accession number (BLAST NCBI)
CI 6	1436	*Virgibacillus salarius*	99%	NR 041270.1
BAFBB 5	1552	*Virgibacillus marismortui*	99%	NR 028873.1

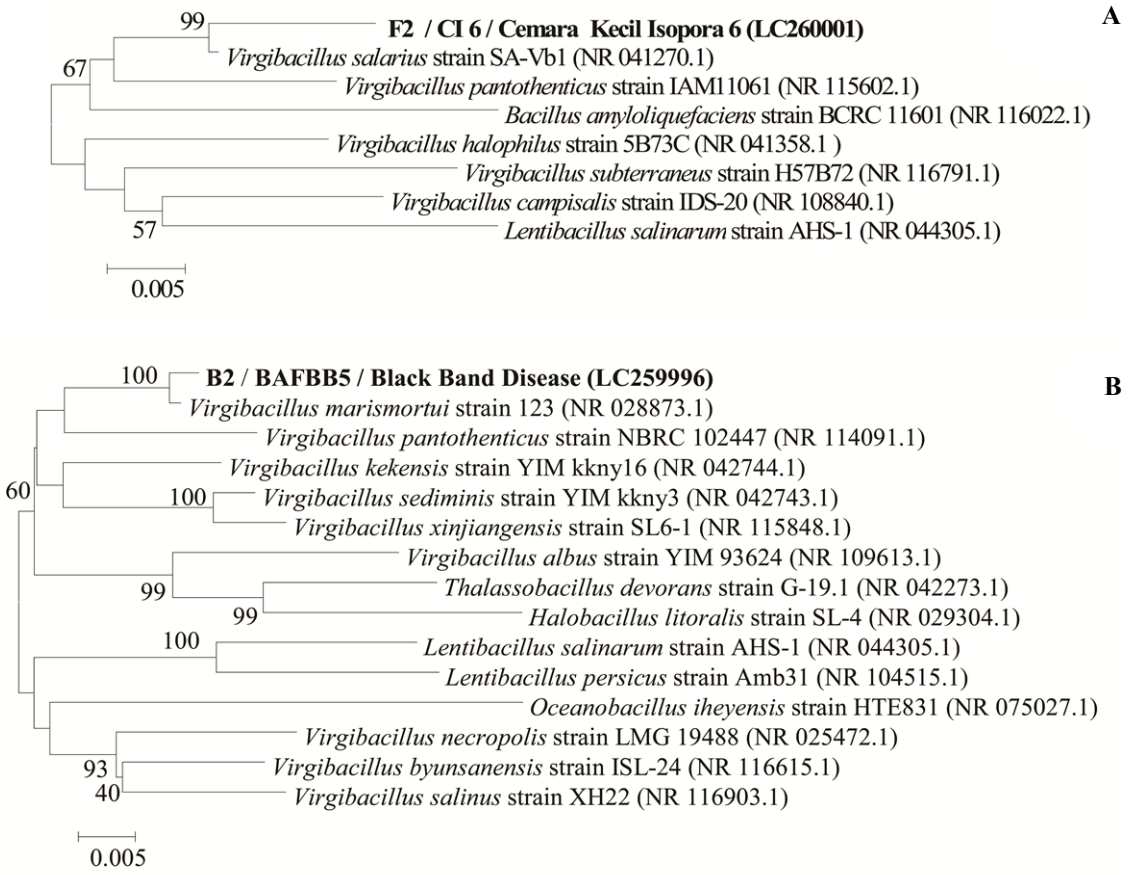

Figure 3. Phylogenetic tree based on the 16S ribosomal DNA sequence data showing the relationships of representative strains with the most closely related bacteria identified in the GenBank database. (A) Showed closest match between CI6 (**LC 260001**), associate bacteria obtained from coral symbiont bacteria that has ability to inhibit growth of BBD associated bacteria, *Virgibacillus salarius* strain SA-Vb1. *Carnobacterium pleistocenium* was used as outgroup. (B) Showed closest relationship between BAFBB 5 (**LC 259996**) associate bacteria that caused a black band disease and *Virgibacillus marismortui* strain 123. *Pseudoalteromonas aliena* was used as outgroup

BBD is the best-studied coral disease with numerous researches ranging from pathogeneses to etiology and epizootiology (Weil and Rogers 2011). Based on their consistently dominant populations in the BBD bacterial mat, Carlton and Richardson (1995) suggested that a microbial consortium consists of a cyanobacterium (*Phormidium corallyticum*), a sulfide-oxidizing bacterium (*Beggiatoa* sp.) and a sulfate-reducing bacterium (*Desulfovibrio* sp.) contribute to the incidence of the BBD. However, growing studies showed that multi etiologic agents are responsible for BBD incidence (Buerger et al. 2016). The BBD microbial community is reported to be varied among region (Weil and Rogers 2011) and depend on host coral species and methodology used (Cooney et al.2002; Frias-Lopez et al. 2004; Sekar et al. 2006). Differ bacterial composition observed spatially or temporally was reported from Florida, Bahamas and the US Virgin (Voss et al. 2007). Barneah et al. (2007) suggested that BBD is a polymicrobial disease. Molecular techniques revealed wider range of microorganisms than previously described (Bourne et al. 2009). Current review on BBD onset suggested that shifting of microbial community triggered the virulence of the disease (Sato et al. 2016).

In this study, 14 isolates out of 87 (approximately 16%) bacteria isolates of healthy corals showed antagonism activity against the BBD isolates. Similar results were obtained from previous study when 34 (18.58%) of cultured strains obtained from healthy corals showed their antimicrobial activity against the BBD strains of *Montipora* sp. (Sabdono et al. 2017). In *Acropora palmata*, almost 20% of the culture bacteria isolated from the coral mucus demonstrated antipathogenic activity, including towards the causative agent of white pox disease (Ritchie, 2006). Lower results were obtained from antipathogenic study of culture strains isolated from *Oculina patagonica*, where only 5.8% among the cultured strains showed antibacterial activity against *Vibrio shiloi*, the known pathogen of the coral (Nissimov et al. 2009). Differ results were reported from the Red Sea. The stony corals showed little or no antibacterial activity against marine bacteria isolated from the seawater compared to 83% of soft coral antimicrobial activity (Kelman et al. 2006). □

Inhibition zones that were observed on the overlay test and the agar diffused test result indicated the possible production of antipathogenic activity of coral symbiont bacterial against the BBD isolate. Antibacterial activity demonstrated by associated-bacteria of healthy coral is likely showed beneficial relationship among resident of microbial community that might be useful for coral protection against pathogenic bacteria (Weir and Rogers, 2011). However, its mechanism needs further investigation. Krediet et al. (2012) suggested that coral commensal bacteria may block the expansion of opportunistic pathogens though metabolic interaction was not clear. In this study, *V. salarius* which showed antibacterial activity against the BBD strain was obtained from the *A. muricata* that suffered from the disease. Gantar et al. (2011) demonstrated that BBD cyanobacteria played a significant role in structuring the multiple partite of BBD microbial community by production of antimicrobial compounds.

Characterization of bacteria associated with the disease as well as antipathogenic data was already reported from Karimunjawa Island. Various strains of bacteria, member of Phylum Bacteriodetes and γ-proteobacteria were reported to be associated with BBD (Sabdono and Radjasa 2006a; Sabdono et al. 2017). Phylogenetic analysis shows that associated bacteria CI 6 obtained from healthy coral that have antipathogenic ability is closely related to *Virgibacillus salarius*, a gram-positive, endospore-forming, rod-shaped and moderately halophilic bacterium isolated from Saharan salt lake (Hua et al. 2008). While the BBD associate bacteria, BAFBB 5 isolate has close relationship with *V. marismortui*, a halophilic bacteria isolated from Dead Sea, a member of Phylum Firmicutes (Arahal et al. 1999). Both bacteria are member of genus *Virgibacillus*, belongs to family Bacillaceae within phylum Firmicutes. The members of the genus commonly found in many habitats and mostly are isolated from saline environments (Sánchez-Porro et al. 2014). Barneah *et al*. (2007) reported that Firmicutes bacteria was among bacterial group that found at the adjacent area of coral tissues that affected by BBD. Firmicutes was also known as common bacterial group found in mucus of healthy corals, microbial mat of BBD and surface of death coral after attacked by BBD (Frias-lopez et al. 2002). However, none of bacteria that caused the BBD infection at Karimunjawa Islands are same as currently reported as causative agent of BBD from the Caribbean. It is likely that wide range of microbial community is responsible for BBD infection of corals as reported previously (Barneah et al. 2007; Bourne et al. 2009; Weil and Rogers, 2011; Buerger et al. 2016).

In conclusion, the diverse acroporid corals-isolated bacteria from the Cemara Kecil island, Karimunjawa have limited metabolites with different strength of antibacterial activities. The presence of Firmicutes bacteria against Firmicutes strain may play significant roles in structuring microbial community at the corals and provide antipathogenic activity against BBD bacterial strains. These bacterial group can be of potential use to biocontrol agents of BBD coral disease at least at Karimunjawa Islands.

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