Jurnal of Coastal Development
Volume 9, Number 3, June 2006 : 175 - 182
ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

ANTI-BACTERIAL PROPERTY OF A CORAL-ASSOCIATED BACTERIUM *Bacillus* sp. AGAINST CORAL PATHOGENIC BBD (*BLACK BAND DISEASE*)

Agus Sabdono [1,2*] *and Ocky Karna Radjasa*[1,2]

[1]Department of Marine Science, Diponegoro University, Semarang – 50275, Central Java, Indonesia
[2]Center for Tropical Coastal and Marine Studies, Diponegoro University, Widya Puraya, Semarang – 50275, Central Java, Indonesia

Received : March 5, 2006 ; Accepted : May 10, 2006

ABSTRACT

Marine organisms such as corals are frequently colonized by bacteria that may be pathogenic to them. One of the means by which they are able to combat microbial attack is by chemical defense. A number of metabolites obtained from algae and invertebrates may be produced by associated microorganisms. The purpose of study was to isolate and characterize of coral-associated bacteria having antibacterial potency against BBD coral disease. A coral-associated bacterium, KM2 isolate, was successfully screened for antibacteria production against indigenous BBD pathogenic bacteria based on PCR amplification of the non-ribosomal peptide synthetase gene and was identified as closely related to Bacilus sp based on its 16S rDNA.. KM2 strain was found to inhibit the growth of coral pathogenic BBD bacteria tested Myroides odoratimimus strain BBD1, Bacillus algicola Strain BBD2 and Marine Alcaligenes bacterium Strain BBD3. This bacterium was found to inhibit the growth of all those BBD coral pathogenic bacteria.

Keywords: coral-associated bacterium, molecular characterization, antibacterial activity, *Bacillus sp*

Corespondence : Phone: 024-7474698/7460038; Fax:024-7474698; E-mail: agus_sabdono@yahoo.com

INTRODUCTION

Infectious disease in coral reefs has come out as one of the primary causes of the destruction of coral reef ecosystems (Harvell et al., 1998; Santavy and Peters, 1997; Goreau et al., 1998; Hayes and Goreau, 1998). Black band disease (BBD) is one of the most widespread and destructive of these coral infections (Richardson, 1998). The indicative symptom of BBD is the development of a narrow 0.1- to 7-cm-wide ring-shaped black to red microbial mat that migrates from top to bottom across massive coral colonies, killing healthy coral tissue at rates of as much as 1 cm per day (Richardson, 1996). BBD preferentially affects corals such as *Montastrea annularis*, *Montastrea cavernosa*, and *Diploria strigosa* (Lopez, 2002; Edmunds, 1991; Rutzler and Santavy, 1983). These species, known as framework building corals, form large structures that become the dominant physical elements of reefs (Lopez, 2002). As a result, coral mortality caused by BBD is a potent force in restructuring coral reef ecosystems (Edmunds, 1991).

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

It is a widely observed phenomenon that microbial cells attach firmly to almost any surface submerged in marine environments, grow, reproduce, and produce extracellular polymers that provide structure to the assemblage termed as biofilm (Kioerboe et al. 2003). Furthermore, it is well understood that corals harbor diverse microbial communities (William et al, 1987; Shashar et al, 1994; Kim, 1994; Kushmaro et al, 1996; Rohwer et al, 2001). Their surface is covered by muco-polysaccharides, which provides a matrix for bacterial colonization leading to the formation of biofilm-forming microbial communities (Kushmaro et al, 1997).

Recently many coral-associated bacteria have been characterized as sources of marine natural products (Moore, 1999), especially since the coral surface is more nutrient rich than seawater or even sediments (Unson et al, 1994; Bultel-Ponce et al, 1999). However, colonization of coral surfaces by bacteria and other microorganisms is mostly nondestructive to corals (Paul et al, 1986; Coffroth, 1990 and Kim, 1994).

Due to the close spatial vicinity of these biofilm-forming bacteria, it can be expected that the indigenous microbial population is adapted to competitive conditions, e.g. for available nutrients and space (Slattery et al, 2001). The production of secondary metabolites is a common adaptation of these bacteria to compete in such microenvironments.

More information on coral-associated bacteria might be desirable, as many of these bacteria serve as sources of secondary metabolites including novel antibiotics. Here, we report on antibacterial property of a secondary metabolite-producing coral bacterium closely related to *Bacillus sp.* against coral pathogenic BBD *M. odoratimimus Bacillus algicola* and *Marine Alcaligenes bacterium*

MATERIALS AND METHODS

Sampling and isolation of coral - associated bacteria

The corals were collected from Menjangan Besar island, Karimunjawa (03º 52' 676" S dan 110º 25' 519" E), North Java Sea, Indonesia by scuba diving and identified as *Porites* sp., *Galaxea fascicularis*, *Pavona* sp., and *Acropora* sp. according to Veron (1988). Upon collection coral fragments were put into sterile plastic bags (Whirl-Pak, Nasco, USA) and immediately brought to the Marine Station of the Diponegoro University where it was rinsed with sterile seawater and scraped off with a sterile knife. The resultant tissues were serially diluted, spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan et al, 2000).

Inhibitory interaction test

Inhibitory interaction test of isolate KM2 against pathogenic *Myroides odoratimimus, Bacillus algicola* and *Marine Alcaligenes bacterium* obtained from previous study (Sabdono and Radjasa, unpublished), was performed by using the agar disk-diffusion method (Conception et al, 1994). 100 µl culture of *V. harveyi* in the logarithmic phase (ca. 10^9 cells ml^{-1}) was spread on to agar medium. Paper disks (Φ 8 mm; Advantec, Toyo Roshi, Ltd, Japan) containing 10 µl of the primer-carrying bacterial strain was placed on the respective agar surface. The plates were then incubated at room temperature for 48 hours. Antibacterial activity was defined by the formation of inhibition zones greater than 9 mm around the paper disk.

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

PCR-based screening of NRPS producing bacterial strain

For PCR analysis, genomic DNA of strain KM2 was taken from cell material on an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-70 °C) and thaw (95 °C). Amplification of peptide synthetase gene fragments was carried out with the degenerated primers A2gamF (5´-AAG GCN GGC GSB GCS TAY STG CC-3´) and A3gamR (5´-TTG GGB IKB CCG GTS GIN CCS GAG GTG-3´) (MWG-Biotech, Ebersberg, Germany) designed from conserved regions of various bacterial peptide synthetase sequences from GenBank (Marahiel *et al.*, 1997). PCR was performed with an Eppendorf Mastercycler (Eppendorf Inc., Germany) as follows: 2 µl template DNA, 40 pmol of each of the appropriate primers, 125 µmol of each deoxyribonucleoside triphosphate, 5 µl of 10 x RedTaq™ PCR buffer (Sigma, Germany), 1.2 mg ml^{-1} (final concentration) bovine serum albumin (Sigma) and 0.75 unit RedTaq™ DNA polymerase (Sigma) were adjusted to a final volume of 50 µl with sterile water (Sigma). A PCR run comprised 40 cycles with denaturing conditions for one minute at 95°C, annealing for one minute at 70 °C and extension for two minutes at 72 °C, respectively.

PCR amplification and sequencing of 16S rRNA gene fragments.

PCR amplification of the partial 16S rRNA gene of strain KM2, purification of PCR products and subsequent sequencing analysis were performed according to the method of Brinkhoff and Muyzer (1997). The determined 394 bp DNA sequence of strain KM2 was then compared for homology to the BLAST database (Altschul, 1997).

PCR amplification and sequencing of 16S rRNA gene fragments.

PCR amplification of the partial 16S rRNA gene of strain KM2, purification of PCR products and subsequent sequencing analysis were performed according to the method of Brinkhoff and Muyzer (1997). The determined 394 bp DNA sequence of strain KM2 was then compared for homology to the BLAST database (Altschul, 1997).

Phylogenetic analysis.

A phylogenetic tree was constructed using maximum-likelihood analysis. Alignment positions at which less than 50 % of sequences of the entire set of data had the same residues were excluded from the calculations to prevent uncertain alignments within highly variable positions of the 16S rDNA. CLUSTAL X was used for multiple alignment/pairwise the DNA sequence (Thompson et al, 1997). Phylogenetic analysis was performed with the PAUP*4.0 (Phylogenetic Analysis Using Parsimony) software package (Swofford, 1998).

RESULTS DAN DISCUSSION

Results

Inhibitory interaction test showed that strain KM2 inhibited the growth of coral pathogenic *M. odoratimimus, B. algicola* and *M. Alcaligenes bacterium* (Table 1; Figure 1).

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

Table 1. Inhibition test of coral bacteria against coral pathogenic BBD

No.	Isolates	*coral pathogenic* BBD:		
		M. odoratimimus	*B. algicola*	*M.A. bacterium*
1.	KM1	-	-	-
2.	KM2	+	+	+
3.	KM3	-	-	-
4.	KM4	-	-	-
5.	KM5	-	-	-
6.	KS1	-	-	-
7.	KS2	-	-	-
8.	KS3	-	-	-
9.	KS4	-	-	-
10.	KF1	+	-	-
11.	KF2	-	-	-
12.	KB1	+	-	-
13.	KB2	-	-	-
14.	KB3	-	-	-
15.	KB4	-	-	-

Figure 1. Anti-bacterial activity of coral bacteria against pathogenic BBD

Both strain KF1 and KB1 inhibited the growth of coral pathogenic *M. odoratimimus* only. PCR-based screening revealed that the coral-associated bacterial strain KM2 was capable of producing secondary metabolites, in particular a non-ribosomal polypeptides. As indicated in Figure 2, bacterial strain KM2 possesses the NRPS gene as represented by the occurrence of a single DNA band similar to the positive control on the agarose gel.

 ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

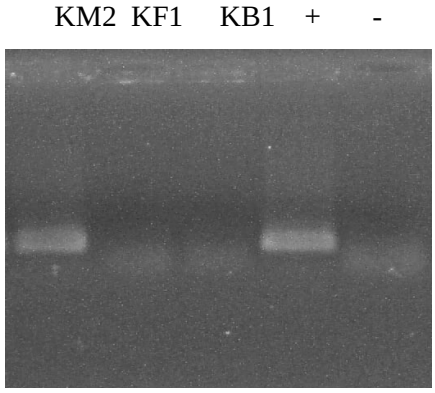

Fig 2. PCR-based screening of NRPS producing-KM2 strain.
+ control (*Pseudomonas fluorescens* DSM No. 50117)

A comparison of the 16S rRNA gene sequence of strain KM2 with sequences from GenBank demonstrated that this strain is affiliated to the family *Bacillus sp*. The phylogenetic tree shown in Figure 3 indicating that isolate KM2 is most closely related with *Bacillus sp.* CNJ941 PL04 *(*accession number DQ448803) with a homology of 99%.

.

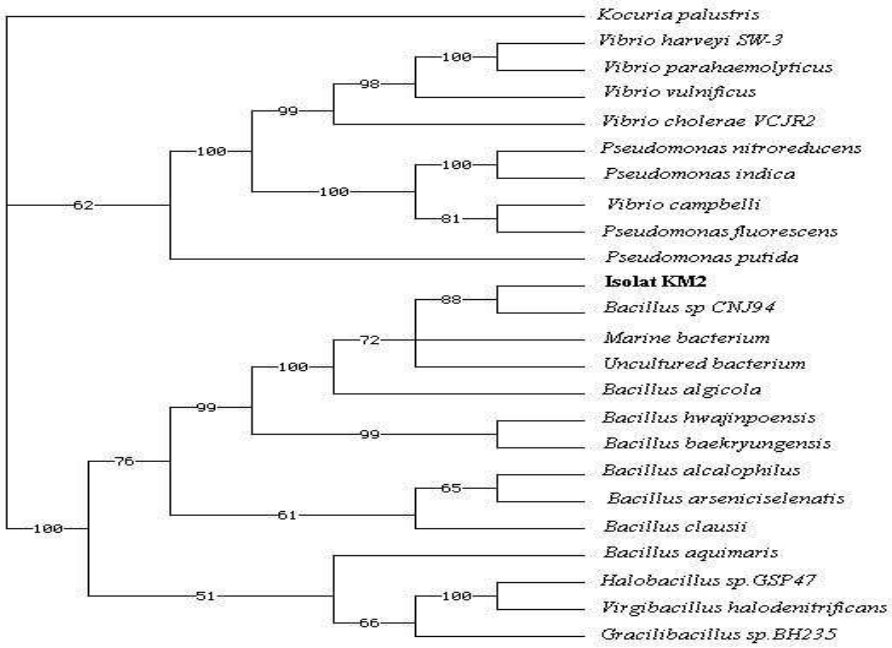

Figure 3. Phylogenetic tree based on comparative 16S rRNA gene sequence analysis of *Bacillus* species showing the phylogenetic affiliation of strain KM2. *Kocuria palustris was used as outgroup.*The bar indicates 2% sequence divergence.

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

Discussion

Inhibitory interactions among coral-associated bacteria that occur on the coral surface are of great interest to search for secondary metabolite-producing bacteria. Isolation and screening for secondary metabolite-producing bacteria in coral reef ecosystems have been strongly neglected until now. Our results highlight one coral-associated bacterium (KM2) carrying the NRPS gene. This bacterium is 99% identical to *Bacillus sp* based on its 16S rRNA gene sequence.

Growth inhibition of *M. odoratimimus, B. algicola* and *M. Alcaligenes bacterium* by NRPS strain KM2 (Table 1) demonstrates the so far uncharacterized secondary metabolites of strain KM2 lead to antagonistic activity and, may hence lead to advantages in the competition for space and nutrients with other coral-associated bacteria. The efficient inhibition of pathogenic bacterium *M. odoratimimus, B. algicola* and *M. Alcaligenes bacterium* by strain KM2 may further reflect the potential role of coral bacteria in controlling coral disease.

Not all proteins are synthesized on ribosomes, and small polypeptides can be assembled by peptide synthetases just as other compounds. Most non-ribosomal peptides from microorganisms are classified as secondary metabolites. They rarely play a role in primary metabolism, such as growth or reproduction but have evolved to somehow benefit the producing organisms (Neilan *et al*, 1999). Products of the microbial non-ribosomal peptide synthesis include the immunosuppressant *cyclosporine* and other antibiotics such as *gramicin S, tyrocin A* and *surfactins* (Kleinkauf and von Dohren, 1996).

Interestingly, the organism closest related to KM2, *Bacillus sp*, owns a non-ribosomal peptide synthetase, which produces the *siderophore alterobactin* (Reid et al., 1993; Deng et al., 1995). Although the biological function of the gene product remains unknown, the feasibility that the respective gene detected in strain KM2 codes for a non ribosomal peptide synthetase is high.

CONCLUSION

The present work highlights the production of secondary metabolites by a symbiotic coral bacterium (KM2) carrying the NRPS gene. The expression of the NRPS gene accounts for the biosynthesis of various natural products with different biological activity (Silakowski et al, 2000). Hence, the application of molecular approach through PCR using specific NRPS primers provides rapid detection and is suitable to greatly improve the screening efficiency for secondary metabolite-producer among coral-associated bacteria against BBD coral pathogenic *M. odoratimimus, B. algicola* and *M. Alcaligenes bacterium.*

ACKNOWLEDGEMENTS

The work was part of a research grant provided by Directorate General of Higher Education within the PENELITIAN DASAR Programme for Agus Sabdono (Contract. No. 16/P2IPT/DPPM/PID/III/2004).

REFERENCES

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman D J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. N*ucleic. Acids*. *Res.* 25: 3389-3402.

Brinkhoff T, Muyzer G. 1997. Increased species diversity and extended habitat

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

range of sulfur-oxidizing *Thiomicrospira* spp. *Appl. Environ. Microbiol.* 63: 3789-3796.

Bultel-Ponce V, Berge J-P, Debitus C, Nicolas J-L, Guyot M. 1999. Metabolites from the sponge-associated bacterium *Pseudomonas* species. *Mar. Biotechnol*. 1: 384-390.

Coffroth MA. 1990. The fucntion and fate mucous sheets produced by reef coelenterates. Procc The 6th Int Coral Reef Symp 2: 15-20. Australia.

Deng J G, Hamada Y, Shioiri T. 1995. Total synthesis of alterobactin-A, a super siderophore from an open-ocean bacterium. *J. Am. Chem. Soc*. 117 (29): 7824-7825.

Edmunds, P. J. 1991. Extent and effect of black band disease on Caribbean reefs. *Coral Reefs* 10:161-165.

Goreau, T. J., J. Cervino, M. Goreau, R. Hayes, M. Hayes, L. Richardson, G. Smith, K. DeMeyer, I. Nagelkerken, F. J. Garzon, D. Gil, G. Garrison, E. H. Williams, W. L. Bunkley, C. Quirolo, K. Patterson, J. W. Porter, and K. Porter. 1998. Rapid spread of diseases in Caribbean coral reefs. *Rev. Biol. Trop*. 46:157-171.

Harvell, C. D., K. Kim, J. M. Burkholder, R. R. Colwell, P. R. Epstein, D. J. Grimes, E. E. Hofmann, E. K. Lipp, A. D. M. E. Osterhause, R. M. Overstreet, J. W. Porter, G. W. Smith, and G. R. Vasta. 1999. Emerging marine diseases—climate links and anthropomorphic factors. *Science* 285:1505-1510.

Hayes, R. L., and N. I. Goreau. 1998. The significance of emerging diseases in the tropical coral reef ecosystem. Rev. Biol. Trop. 46:173-185Kim K. 1994. Antimicrobial activity in gorgonian corals (Coelenterata, Octocorallia). *Coral. Reefs*. 13: 75-80.

Kiorboe T, Grossart HP, Ploug H, Kam T. 2003. Microbial dynamics on particles: colonization, growth, detachment, and grazing mortality of attached bacteria. *Appl. Environ. Microbiol*. 69:3036-3047

Kushmaro A, Loya Y, Fine M, Rossenberg E. 1996. Bacterial infection and coral bleaching. *Nature.* 380: 396.

Madigan MT, Martinko JM, Parker J, Brock TD. 2000. Biology of microorganisms. Prentice-Hall, Inc., Upper Saddle River, New Jersey 07458

Marahiel MA, Stachelhaus T, Mootz HD. 1997. Modular peptide synthetases involved in nonribosomal peptide synthesis. *Chem. Rev.* 97: 2651-2673.

Moore BS. 1999. Biosynthesis of marine natural products : microorganisms and macroalage. *Nat. Prod. Rep*. 16: 653-674.

Munro MHG, Blunt JW, Dumdei EJ, Hickford SJH, Lill RE, Li S, Battershill CN, Duckworth AR. 1999. The discovery and development of marine compounds with pharmaceutical potential. *J. Biotechnol.* 70: 15-25.

Neilan BA, Dittmann, Rouhiainen L, Bass RA, Schaub V, Sivonen K, Borner T .1999. Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. J. *Bacteriol.* 181:4089-4097.

Paul JH, DeFlaun ME, Jefffrey WH. 1986. Elevated levels of microbial activity in the coral surface microlayer. *Mar. Ecol. Prog. Ser*. 33:29-40.

Reid R T, Live DH, Faulkner DJ, Butler A. 1993. A siderophore from a marine bacterium with an exceptional ferric

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

ion affinity constant. *Nature*. 366(6454):455-8.

Richardson, L. L. 1998. Coral disease: what is really known? *Trends Ecol. Evol.* 13:438-443.

Richardson, L. L. 1996. Horizontal and vertical migration patterns of *Phormidium corallyticum* and *Beggiatoa* spp. associated with black-band disease of corals. Microb. Ecol. 32:323-335.[Medline]

Rohwer F, Breitbart M, Jara J, Azam F, Knowlton N. 2001. Diversity of bacteria associated with the Caribean coral *Montastraea franksi.* Coral. Reefs.20:85-95.

Rützler, K., and D. L. Santavy. 1983. The black band disease of Atlantic reef corals. I. Description of the cyanophyte pathogen. Mar. Ecol. 4:301-319.

Santavy, D. L., and E. C. Peters. 1997. Microbial pests: coral disease in the Western Atlantic, p. 607-612. Eighth International Coral Reef Symposium, vol. 1, Balboa, Panama.

Shashar, N., Cohen, Y., loya, Y., and Sar, N. 1994. Nitrogen fixation (acetylene reduction) in stony corals: evidence for coral-bacteria interactions. Mar. Ecol. Prog. Ser. 111: 259-264.

Silakowski, B., G. Nordsiek., B. Kunze., H. Blöker., and R. Müller. 2000. Novel features in a combined polyketide synthase/non-ribosomal peptide synthetase: the myxalamid biosynthetic gene cluster of the myxobacterium *Stigmatella aurantiaca* Sg a15[1.] Chem. Biol. 53:1-11.

Slattery M, Rajbhandari I, Wesson K. 2001. Competition-mediated antibiotic induction in the marine bacterium *Streptomyces tenjimariensis*. Microb. Ecol. 41:90-96.

Swofford, D. L. 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts. Copyright © Smithsonian Institution, 1998.

Unson, M.D., Holland, N.D., Faulkner, D.J. 1994. A brominated secondary metabolite synthesized by the cyanobacterial symbiont of marine sponge and accumulation of the crystalline metabolites in the sponge tissue. Mar. Biol. 119: 1-11.

Williams, W.M., Viner, AB., Broughton, W.J. 1987. Nitrogen fixation (acetylene reduction) associated with the living coral *Acropora variabilis.* Mar. Biol. 94: 531-535.

ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004