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MOLECULAR DIVERSITY OF SECONDARY METABOLITE-PRODUCING MARINE MICROORGANISMS ASSOCIATED WITH INDONESIAN REEF'S INVERTEBRATES

Ocky Karna Radjasa* **and Agus Sabdono**
Center for Tropical Coastal and Marine Studies
Department of Marine Sciences, Diponegoro University, Semarang, 50275, Indonesia
*e-mail: ocky_radjasa@yahoo.com

ABSTRACT

The metabolites from microorganisms are a rapidly growing field, due to the suspicion that a number of metabolites obtained from reef's invertebrates are produced by associated microorganisms. Less than 2% of microbial flora has been successfully isolated from marine environment. Coral reefs are the most diverse marine ecosystems, however, little is known about the microbial diversity in these ecosystems. It is expected that still quite a few parts of unexplored culturable invertebrate-associated microorganisms exists in the reef environments. The present study aimed at estimating the biodiversity of secondary metabolite-producing microbes associated with reef's invertebrates such as coral, soft coral and sponge collected from geographically different areas.

Keywords: Molecular diversity, Microorganisms, Reef, Invertebrate, Secondary metabolites

INTRODUCTION

The oceans are the source of a large group of structurally unique natural products that are mainly accumulated in invertebrates that are common to coral reef ecosystems, such as sponges, tunicates, bryozoans, soft corals and molluscs. Several of these secondary metabolites showed pronounced pharmacological activities and are interesting candidates for new drugs and coral reef ecosystem has been one of the main sources for the search of bioactive compounds (Radjasa, 2003).

Serious obstacle to the ultimate development of most marine natural products that are currently undergoing evaluation and trials is the problem of supply due to their low concentrations (Munro *et al.*, 1999). The concentrations of many highly active compounds in marine invertebrates are often minute, sometimes accounting for less than 10^{-6}% of the wet weight (Procksch *et al.*, 2002).

In addition, it has often proven extremely difficult, and some cases impossible, to provide from invertebrates sufficient amounts of many of these substances due to limited amounts found in the producing organism, or to limited quantity of the organism itself, or to geographic, seasonal or sexual variations in the amounts and in the nature of produced secondary metabolites (Kelecom, 2002).

Regarding the use of reef's invertebrates as sources of bioactive compounds, it has been primarily concerned with protection of the environment and sustainability. The ecological ethics need to be taken into account, especially in the light of the importance of coral reefs for human population in tropical coastal communities. Thus, considering bioethical perspectives and finding alternative solutions to the problem supply for bioactive compounds mainly produced by reef's invertebrates should be given high priority.

Many marine species have been collected in the search for novel bioactive compounds and for developing pharmaceutical drugs (Hooper *et al.*, 1998; Quinn *et al.*, 2002). The collections tend, however, to focus on organisms containing chemicals, known as secondary metabolites that primarily serve ecological functions in competition for space and in protection from predation, fouling,

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and ultraviolet light (Harper *et al.*, 2001), as well from bacterial infections (Correa, 1997; Rohwer *et al.*, 2002) To date, the majority of novel compounds have been secondary metabolites from soft-bodied, sessile invertebrates, such as Porifera (sponges); Cnidaria (jellyfish, corals, sea anemones); and Urochordata (ascidians).

To date, the primary target for marine bioprospecting has been tropical seas. Coral reefs have been targeted for bioprospecting because they host a high level of biodiversity and are often characterised by intense competition for space, leading to a chemical warfare among sessile organisms.

The collections tend, however, to focus on organisms containing chemicals, known as secondary metabolites that primarily serve ecological functions in competition for space and in protection from predation, fouling, and ultraviolet light as well from bacterial infections (Pawlik *et al.*, 2002; Becerro *et al.*, 1997; Hellio *et al.*, 2005). The occurrence of large scale of bioactive compounds is not common to all living organisms, but restricted to certain taxonomic groups. Among marine animals, reef's invertebrates are the most prolific producers of secondary metabolites.

Perhaps the most significant problem that has hampered the investigation of secondary metabolites is their low concentration. In marine invertebrates many highly active compounds contribute to $<10^{-6}$ % of the body-wet weight (Proksch *et al.*, 2002; Radjasa *et al.*, 2007).

There has an increasing concerns regarding the collecting reef's organisms for the discovery and development of pharmaceuticals since it has been perceived variously as sustaining and threatening conservation. There is an urgent need to take into account the bioethical considerations in anticipating the potential consequences of these activities and proposing management options for sustainable use of reef's invertebrates as the sources of bioactive compounds (Sukarmi and Radjasa, 2007).

Understanding of marine invertebrate-microbial associations is a fundamental step in studying biologically potential active, possible medicinal compounds from associated bacteria. In particular, from sustainability point of view, isolating bioactive-producing bacteria or their genes involved in the biosynthesis of bioactive compounds is obviously offers a much better approach than cultivating and harvest invertebrates, which are in most cases extremely difficult.

Recently, PCR amplification of degenerate primers targeted to sequences of genes essential in the biosynthesis of particular secondary metabolites has been used to estimate the genetic ability of reef's microorganisms to produce compounds belonging to Non-ribisomal peptide syntethases (NRPS), and polyketide synthases (PKS) (Radjasa and Sabdono, 2003; Radjasa *et al.*, 2007a, b).

The present work is aimed at screening of marine bacteria associated with reef's invertebrates for the production of secondary metabolites against indicator organisms coupled with PCR-based analysis for the presence of PKS and NRPS of the active isolates followed by subsequent DNA sequencings.

MATERIALS AND METHODS

Sampling and isolation of soft coral-associated bacteria

Colonies of coral were collected from Teluk Awur water, Jepara, North Java from a depth of five meters. Colonies of softcoral were collected from Peucang Island, West Java from a depth of three meters. Sponge colonies were obtained from Menjangan Kecil Island, Karimunjawa, Java Sea from a depth of five meters. All specimen collections were performed by scuba diving. Upon collection soft coral, coral and sponge colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA). The tissues were then 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).

Isolation of marine biofilm-forming bacteria

Isolation was carried out with a method modified from Harder *et al.* (2003). Four pre-sterilized glass slides had been deployed in four different places around soft coral colony for a week. The biofilm developed in these glass slides were then put into sterile petri dish, rinsed with sterile seawater and scrapped off with a sterile knife. The resultant mixture was diluted. One hundred µl of each dilution was spreaded onto ½

strength ZoBell2216E and incubated at room temperature for 48 hours. Colonies with distinguished feature were selected and purified.

Screening of coral bacteria with biological activity

To screen their biological activity, bacterial isolates were tested against marine biofilm forming bacteria, and pathogenic bacteria. One 100 µl culture of each indicator microorganism in the logarithmic phase (ca. 10^9 cells ml^{-1}) was spread on to agar medium. Several paper disks (8 mm; Advantec, Toyo Roshi, Ltd, Japan) containing 10 µl of invertebrate bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 hours. Biological activity was defined by the formation of inhibition zones around the paper disk. Isolates showed biological activity against indicator organisms were chosen for further screening based on PCR technique by using specific primers of Non-ribosomal peptide synthetase (NRPS) and Polyketide synthases (PKS).

PCR-based analysis of NRPS and PKS producing bacterial strains

To obtain genomic DNA of secondary metabolite producing-strains for PCR analysis, cell materials were taken from an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C) (Radjasa *et al.*, 2007c). Amplification of peptide synthetase gene fragments was carried out with the NRPS 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′) (Marahiel *et al.*, 1997) and PKS degenerated primers KSDPQQF (5′-MGN GAR GCN NWN SMN ATG GAY CCN CAR CAN MG-3′) and KSHGTGR (5′-GGR TCN CCN ARN SWN GTN CCN GTN CCR TG -3′) (Piel, 2002). The NRPS-PCR run comprised 40 cycles with denaturing conditions for 1 min at 95°C, annealing for 1 min at 70°C and extension for 2 min at 72°C, respectively. P*seudomonas* sp. DSM 50117 was used as positive control. The amplification of PKS gene fragments included an initial denaturating step at 94°C for 2 min, followed by 45 cycles at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min. *Bacillus subtilis* 168 was utilized for positive control.

Cloning and sequencing of (putative) peptide synthetase and polyketide domains

The amplified PCR-products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturers protocol. The Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used for subsequent sequencing on an ABI 310 analyzer (Perkin Elmer Applied Biosystems, Foster City, USA).

Rapid grouping of sponge isolates by using Rep-PCR

Rapid grouping among sponge active isolates was carried out by using rep-PCR. The method was performed according to Radjasa *et al.* (2007c, d).

PCR amplification and sequencing of 16S rRNA gene fragments.

PCR amplification of partial 16S rRNA gene of active strains, purification of PCR products and subsequent sequencing analysis were performed according to the method of Thiel and Imhoff (2003). The partial 16S rDNA sequences of strains were compared for homology to the NCBI GenBank and EMBL databases using BLAST (Basic Local Alignment Search Tool).

RESULTS

Screening among marine bacteria associated with softcoral *Sinularia* sp. by using test organism of marine biofilm-forming bacteria (Table 1) revealed that four isolates were capable of inhibiting the growth of marine bioflim forming bacteria (Table 1).

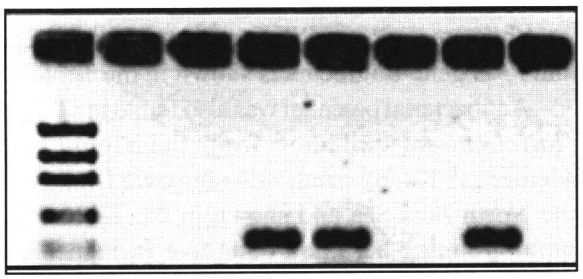

Figure 1. PCR amplification of NRPS gene fragments of softcoral bacteria SFNB.3 and TASF1.2. + control *Pseudomonas* sp. DSM 50117.

Table 1. Characterization of marine biofilm forming bacteria.

No	Strain	Closest relative	Homology (%)	Accession number
1	BIO1.1	*Pseudoalteromonas* sp.	99	AJ391202
2	BIO1.7	*Vibrio harveyi*	97	AJ672389
3	BIO1.8	*Staphylococcus sciuri*	97	S83569
4	BIO2.6	*Tenacibaculum marilutum*	97	AY661693

Table 2. Inhibitory interaction of softcoral bacteria against biofilm-forming bacteria.

Strain	Closest relative	Marine biofilm-forming bacteria			
		BIO1.1	BIO1.7	BIO1.8	BIO2.6
SFNB.3	*Arthrobacter nicotianae*	-	+	-	+
PPSF3.7	*Pelagiobacter variabilis*	-	+	+	+
TASF4.3	*Shewanella alga*	+	+	-	-
TASF1.2	*Pseudomonas fluorescens*	-	-	+	-

Table 3. Characterization of the NRPS gene fragments of softcoral bacteria.

Strain	Length (bp)	Closest gene fragment
SFNB.3	416	*Actinoplanes teichomyceticus* tecR gene, ORF's 1 & 2 (62.46%). Acc. No. (X56928)
TASF1.2	389	putative acetylase, pvdS, putativethioesterase, and pyoverdine synthetase A (pvsA) genes (77%)Acc. AF237701

PCR-based screening showed that two softcoral isolates amplified NRPS gene fragments as indicated in the Figure 1. Following cloning and sequencing of the NRPS products, the results showed that both NRPS products had homology to peptide synthetase gene fragments (Table 3).

Antibacterial activity was also shown by coral bacteria as indicated in the Table 5.

One of coral bacteria was able to amplify the NRPS gene fragment (BJB) and another one, BJ.7 amplified PKS gene fragment (Fig. 2 and 3). Characterization of the produced both NRPS and PKS gene fragments is shown in the Table 4.

Antibacterial potential was also found in sponge *Haliclona* sp. collected from the vicinity of Menjangan Kecil Island, Karimunjawa Islands of the North Java Sea and shown in the Table 6. A rapid grouping based on repetitive PCR method was carried out to group the active isolates (Fig. 4). Six isolates were selected for further DNA sequencing. The identification of the representative isolates is shown in the Table 6.

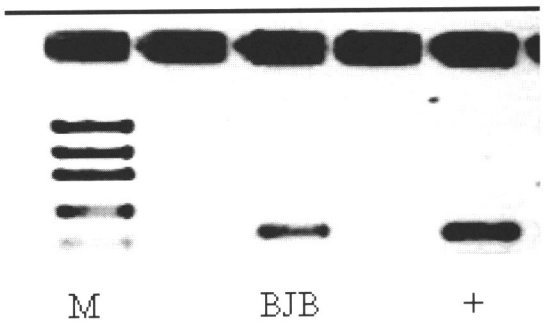

Figure 2. PCR amplification of NRPS gene fragment of coral bacterium BJB. + control *Pseudomonas* sp. DSM 50117.

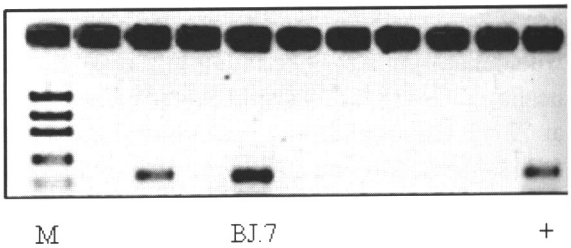

Figure 3. PCR amplification of PKS gene fragments of coral bacterium BJ.7; + control *Bacillus subtilis* 168.

Table 4. Characterization of NRPS and PKS gene fragments.

Strain	PKS/NRPS	Closest gene fragment	Homology (%)	Acc.number
BJB	PKS	Polyketide synthase of *B. subtilis*	73	U11039
BJ	NRPS	Peptide synthetase of *Actinoplanes teichomyceticus*	62.4	X56928

Table 5. Antibacterial activity of coral-associated bacteria.

Strain	Closest relative	Host coral	Tested bacteria		
			E.coli	*B. subtilis*	*S. lentus*
BJ.7	*Bacillus subtilis*	*Acropora* sp.	+	+	+
BJB	*Micrococcus* sp.	*Acropora* sp.	-	+	+
MJ.11	*Vibrio parahaemolyticus*	*Porites lutea*	-	+	+
U2SB.7	*Bacillus aquamaris*	*Galaxea fascicularis*	-	+	+

Table 6. Antibacterial activity of sponge-associated bacteria against pathogenic bacteria.

No	Strain	Test indicator		
		V. parahaemolyticus	*A. hydrophila*	*S. aureus*
1	BSP11.7	+	+	+
2	BSP11.9	+	+	+
3	BSP12.2	+	+	+
4	BSP12.3	+	-	+
5	BSP5.7	+	+	+
6	BSP11.3/A	+	+	+
7	BSP10.6	+	+	-
8	BSP12.3/A	+	+	-
9	BSP11.3	+	+	-
10	BSP12.1	+	+	-
11	BSP3.4/A	+	+	-
12	BSP1.12	-	+	-
13	BSP7.3A	-	+	-

Table 7. Identification of bacterial isolates associated with sponges.

No	Strain	Closest relative	Homology (%)	Acc. Number
1	BSP11.7	Alpha proteobacterium Z143-1	99	AY762960
2	BSP12.3A	*Brachybacterium rhamnosum*	98	AJ414376
3	BSP5.7	Uncultured bacterium clone TCc-18	98	DQ791467
4	BSP12.1	*Pseudoalteromonas* sp. JL-96	98	AY745871
5	BSP1.12	*Vibrio parahaemolyticus*	99	AF388390
6	BSP11.3	Uncultured alpha bacterium	99	AJ810662

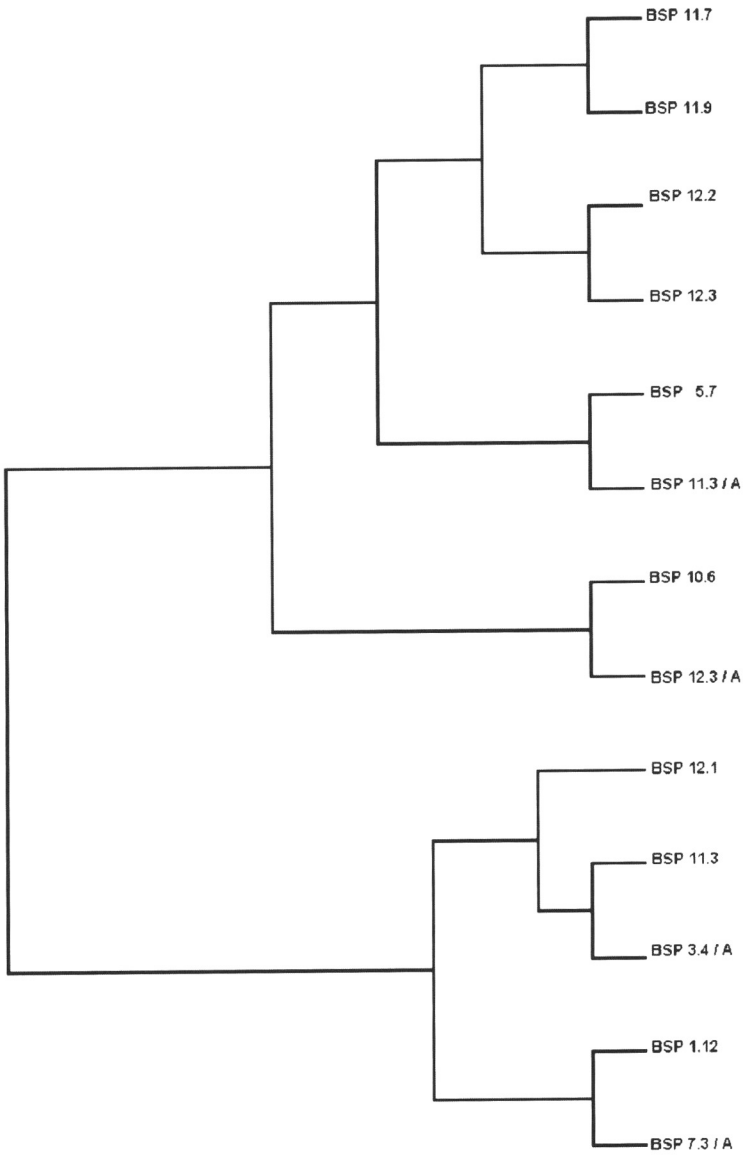

Figure 4. Dendrogram constructed based on rep-PCR of sponge isolates.

DISCUSSION

One of the most interesting aspects of the soft corals is that fouling organisms usually do not colonize their surfaces and it is believed that antifouling represents another ecological role of secondary metabolite in the alcyonacean (Sammarco and Coll, 1992). The present study showed the potential of softcoral-associated bacteria as an alternative antifoulant, in which four isolates were able to inhibit the growth of marine biofilm-forming bacteria isolated from the surrounding colonies of softcoral *Sinularia* sp. Furthermore, two isolates were capable of amplifying the NRPS gene fragments which after cloning and sequencing showed the homology to the peptide synthetases.

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 four coral-associated bacteria inhibited the growth of tested bacteria (Table 5). Furthermore, one isolate carrying the NRPS and another one carrying the PKS gene fragments.

Sponges (phylum Porifera) are most primitive of the multicelled animals that have existed for 700–800 million years. Of the approximately 15,000 sponge species, most occur in marine environments. Only about 1% of the species inhabits freshwater (*Belarbi et al.*, 2003).

It has been known that sponges produce secondary metabolites to repel and deter predators (Becerro *et al.*, 1997), and for communication and protection against infection. In addition, potentially therapeutic compounds identified in sponges include anticancer agents and immunomodulators. Some sponges seem to produce potentially useful antifouling agents (Hellio *et al.*, 2005).

From the present study, it is interesting to note that collected sponge *Haliclona* sp. offered potensial sources of secondary metabolite-producing bacteria as indicated by the results of actibacterial tests against indicator microorganisms *Aeromonas hydrophila*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*.

The application of molecular approach through PCR using specific NRPS and PKS primers provides detection of microorganisms with high pharmaceutical potential and is suitable to greatly improve the screening efficiency for secondary metabolite-producer among reef's invertebrate-associated bacteria.

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