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Richness of Secondary Metabolite-Producing Marine Bacteria Associated with Sponge *Haliclona* sp.

[1,2]Ocky Karna Radjasa, [1,2]Agus Sabdono, [1]Junaidi and [3]Elena Zocchi
[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
[3]Department of Experimental Medicine, Faculty of Medicine and Surgery, University of Genova, Genova, Italy

Abstract: A total of 8 bacterial isolates associated with sponge *Haliclona* sp. collected from Bandengan water, Jepara, North Java Sea, Indonesia, was successfully screened for antibacterial activity against pathogenic bacteria *Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Staphylococcus aureus*. Active bacterial isolates were rapidly grouped by using rep-PCR and a dendrogram was constructed. Five isolates were selected based on the constructed dendrogram for subsequent DNA sequencings resulted in the richness of secondary metabolite-producing sponge associated-bacteria having closest similarity to *Vibrio parahaemolyticus*, *Pseudovibrio denitrificans*, *Pseudoalteromonas* sp., alpha proteobacterium and uncultured bacterium clone. The present study highlighted the repetitive-PCR method as a powerful tool for estimating the richness of secondary metabolite-producing parts among sponge colonizers.

Key words: Richness, rep-PCR, sponge-associated bacteria, secondary metabolite, *Haliclona*

INTRODUCTION

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 and have become sources of great interest to natural product chemistry, since they provide a large proportion of bioactive compounds with different biological activities.

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 (Pawlik *et al*., 2002), compete for space with other sessile species (Davis *et al*., 1991; 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).

Recent research progresses reported that many bioactive natural products from marine invertebrates have striking similarities to metabolites of their associated microorganisms including bacteria (Proksch *et al*., 2002; Thiel and Imhoff, 2003; Radjasa *et al*., 2007a). Thus, it is important to highlight the possible role of marine bacteria associated with sponges in providing solution to the problem of infection by pathogenic bacteria. Bacteria-sponge association that occurs on the sponges then could be of great interest to search for potential use as new source of antibiotics in particular as a solution of the problem of supply of most bioactive compounds produced by reef's invertebrates.

Advanced techniques of molecular biology such as Polymerase Chain Reaction (PCR), has played important roles in estimating the richness of marine microorganisms. Urakawa *et al*. (1999a and b) and Radjasa *et al*. (2001) used Restriction Fragment Length Polymorphism (RFLP) technique for rapid grouping of large number of marine pyschrophilic and psychrotrophic isolates. Very recently, Radjasa *et al*. (2007c) successfully applied repetitive-PCR for grouping marine psychrotrophic bacteria collected from deep-sea waters of Makasar strait, Indonesia. To our knowledge, this technique has not been employed to

Corresponding Author: Ocky Karna Radjasa, Center for Tropical Coastal and Marine Studies, Diponegoro University, Widya Puraya, Semarang, 50275, Central Java, Indonesia
Tel: +62-24-7460038/7474698 Fax: +62-24-7460039/7474698

estimate the richness of secondary metabolite-producing bacteria especially those associated with reef's invertebrates.

The present research is aimed at screening of marine bacteria associated with sponge *Haliclona* sp. for the production of secondary metabolites against pathogenic *Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Stahylococcus aureus* coupled with rep-PCR for rapid grouping of the active isolates followed by subsequent DNA sequencings.

MATERIALS AND METHODS

Sampling and isolation of sponge-associated bacteria: Colonies of sponge *Haliclona* sp. were collected from Bandengan water, Jepara, North Java, Indonesia by scuba diving from a depth of approximately 3 m. Upon collection sponge colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA) and brought to Marine Station, Diponegoro University. Isolation was carried out at the Marine Microbiology Laboratory, Department of Marine Science, Diponegoro University, Semarang, Indonesia. The sponges were then rinsed with sterile seawater and 1 cubic centimeter of sponge tissue was excised from the middle of the whole sponge and sponge surface after it is peeled off (Kim *et al*., 2005) 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 h. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al*., 2000).

Screening of coral bacteria with biological activity: Screening and antibacterial tests as well as molecular based-works were carried out at Marine Microbiology Laboratory, Diponegoro University, Semarang, Indonesia. To screen their biological activity, a total of 56 sponge isolates were tested against isolate pathogenic bacteria *Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Staphylococcus aureus*. One hundred microliter 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 30 μL of the coral bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 h. Antibacterial activity was defined according to Radjasa *et al.* (2007a) by the formation of inhibition zones greater than 9 mm around the paper disk.

Repetitive-PCR: Molecular based-works including DNA extractions, rep-PCR and PCR were carried out at the Laboratory of Marine Biotechnology, Department of Marine Science, Diponegoro University. Whereas, the DNA sequencings were performed at the Molecular Biology Laboratory, Agency for the Assessment and Application Technology (BPPT), in Jakarta, Indonesia.

For rep-PCR, BOX A1R (5'-CTACggCAAggCgACgCTgACg-3') (Versalovic *et al*., 1994) were used. The REP 1 R-I and REP 2-I primers contain the nucleotide inosine (I) at ambiguous positions in the REP consensus (34). PCR reaction contained of 1 μL DNA template (diluted 100x), 1 μL primer, 7, 5 μL Megamix Royal dan sterile water up to total volume of 15 μL. Amplifications were performed with a thermal cycler model Gene Amp PCR System 9700 with the following temperature profiles: initial denaturation at 95°C for 5 min 30 cycles of denaturation (92°C for 1 min), annealing (50°C for 1, 5 min), extension (68°C for 8 min) and final extension at 68°C for 10 min. Five microliter aliquot PCR products were run using elektrophores on 6% acrilamide gel by using 1 x TBE buffer.

Grouping of isolates: Grouping was carried out as previously described (Radjasa *et al*., 2007c) by making matrixes from the positions of bands on the gel which were then analyzed by using Free Tree program by using UPGMA method for constructing the tree. Resampling was performed by bootstrapping with 1000 replications.

PCR amplification and sequencing of 16S rRNA gene fragments: Amplification was conducted according to method of Radjasa *et al.* (2007a). Genomic DNA of secondary metabolite producing-strains for PCR analysis were obtained from cell materials taken from an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80°C) and thaw (95°C). PCR amplification of partial 16S rRNA gene of sponge bacteria, purification of PCR products and subsequent sequencing analysis were performed according to the method of Radjasa *et al.* (2007b). The determined DNA sequences of strains were then compared for homology to the BLAST database.

RESULTS AND DISCUSSION

Screening among 56 marine bacteria associated with sponge *Haliclona* sp. by using test organism revealed that 8 isolates capable of inhibiting the growth of at least 1 tested indicator, while the rest of isolates showed no activity. The measurement of inhibition zone as indicator of the antibacterial potential of active isolates against pathogenic bacteria is presented in the Table 1.

The result of grouping based on rep-PCR technique is presented in the Fig. 1.

Table 1: Antibacterial activity of sponge bacteria against pathogenic bacteria

	Pathogenic bacteria		
Isolates	*A. hydrophila*	*S. aureus*	*V. parahaemolyticus*
BSP1.12	+	-	-
BSP2.7	+	+	+
BSP3.6	+	-	+
BSP4.3	+	+	+
BSP4.7	+	+	-
BSP4.9	+	+	+
BSP5.1	+	-	+
BSP5.2	-	-	+

Sign +: Positive result (diameter of inhibition zone greater than 9 mm); -: Negative result (no inhibition zone)

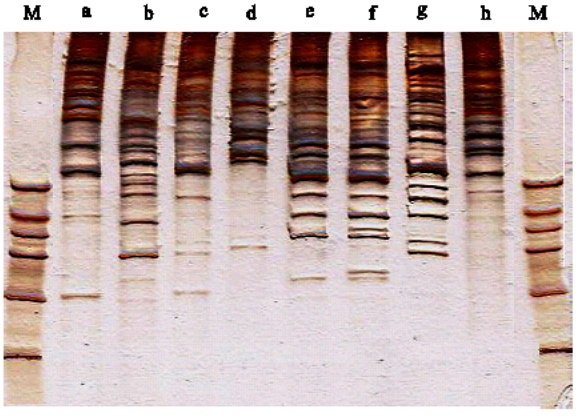

Fig. 1: Rep-PCR analysis of active sponge associated-bacteria M: Marker; a: BSP1.12; b: BSP2.7; c: BSP3.6; d: BSP4.3; e: BSP4.7; f: BSP4.9; g: BSP5.1; h: BSP5.2

Based on the repetitive-PCR results and the constructed dendrogram of the isolates, 5 different isolates representing different groups (Fig. 2) were further selected for DNA sequencings.

A comparison of the 16S rRNA gene sequences of active isolates with sequences from GenBank is shown in the following Table 2.

Perhaps the most significant problem that has hampered the investigation of secondary metabolites produced by reef's invertebrates is their low concentration. In marine invertebrates many highly active compounds contribute to<10^{-6} % of the body-wet weight. Providing sufficient amounts of these biologically active substances, hence, may be a difficult task (Proksch *et al*., 2002; Radjasa *et al*., 2007a, b).

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.

Table 2: Molecular identification of active sponge bacteria

Strains	Closest relative	Homology (%)	Acc. No.
BSP1.12	*Vibrio parahaemolyticus*	99	DQ026024
BSP5.1	*Pseudoalteromonas flavipulchra* JL-96	98	AF297958
BSP2.7	Uncultured bacterium clone TCc-18	98	DQ791467
BSP4.3	Alpha proteobacterium Z143-1	98	AY762960
BSP4.7	*Pseudovibrio denitrificans* DN34	99	AY486423

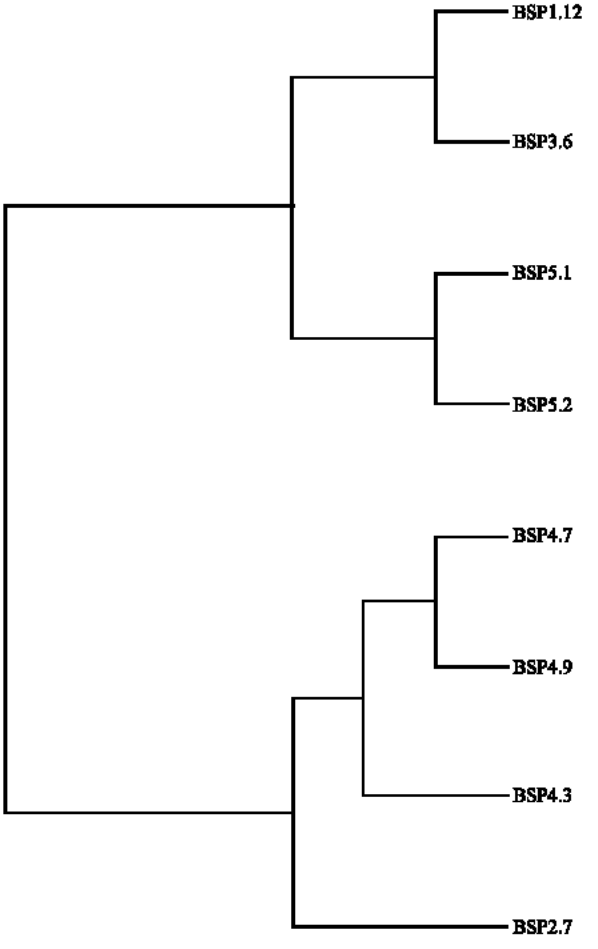

Fig. 2: Dendrogram constructed from banding gel of rep-PCR

There has been an increasing concern 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 potential consequences of these activities and proposing management options for sustainable use of reef's invertebrates as the sources of bioactive compounds.

The present study indicated that marine bacteria associated with sponge *Haliclona* sp. showed strong growth inhibition against indicator microorganisms

(Table 1). This offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria such as *Vibrio parahaemolyticus*, *Aeromonas hydrophila* and *Staphylococcus aureus*.

In this study one isolate, BSP4.7 showing closest relative to *Pseudovibrio denitrificans* (Table 2) inhibited the growth of *A. hydrophila* and *S. aureus* but not *V. parahaemolyticus* (Table 1). A bacterium TA1.6 isolated from the branching coral *Acropora* sp. from neighboring area of the present research was found to be *Pseudovibrio denitrificans* that inhibited the growth of *Bacillus subtilis* (Radjasa, unpublished). Very little reports have documented on the antimicrobial activity of the member of *Pseudovibrio* since *Pseudovibrio denitrificans* was validated as the new genus and species (Shieh *et al*., 2004).

The members of Alteromonadales and Vibrionales of the proteobacteria, such as *Pseudoalteromonas* and *Vibrio* have been known as the dominant antibiotics producers (Long and Azam, 2001; Grossart *et al*., 2004). Futhermore, Radjasa *et al*. (2007a) isolated a coral-associated bacterium TAB4.2 which showed 98% identity to *Pseudoalteromonas luteoviolacea*, an antibiotic-producing bacterium (McCarthy *et al*., 1985; Hanefeld *et al*., 1994) and exhibited growth inhibition against both coral bacteria and pathogenic bacteria. Species of *Pseudoalteromonas* have also been isolated from tunicates (Holmstrom, 1998) and sponges (Ivanova *et al*., 2002).

The present study also showed a bacterium BSP1.12 that closely related to *Vibrio parahaemolyticus* (Table 2) and inhibited only the growth of the tested bacterium *A. hydrophila* (Table 1) known as the causative agent of the disease Motile Aeromonas Septicemia (MAS) in many freshwater fishes. A marine bacterium MJ.11 isolated from massive coral *Porites lutea* of the North Java Sea was found to inhibit the growth of tested bacteria *Bacillus subtilis* and *Staphylococcus lentus* (Radjasa, unpublished).

One isolate obtained BSP2.7 showed closest similarity to uncultured bacterium clone. Considering only about 1% of marine microorganisms have been successfully cultivated, it is not surprising that present work revealed the occurrence of isolate related to the unculturable part of microbial community associated with sponge *Haliclona* sp.

Sponge bacterium BSP4.3 showed high homology to Alpha proteobacterium Z143-1 (98%), a bacterium isolated from Philippine tunicate that produce anti-*Staphylococcus aureus* metabolite heptylprodigiosin (de Guzman, unpublished). Interestingly our isolate BSP4.3 also inhibited the growth of tested strain *Staphylococcus aureus* in addition to *V. parahaemolyticus* and *A. hydrophila* (Table 1).

In conclusion, sponge *Halilona* sp. exhibited diverse secondary metabolite producing-marine bacteria with antibacterial potential against tested strains. The present study highlighted the repetitive-PCR method as a powerful tool in estimating the richness of secondary metabolite producers among colonizers of sponge *Haliclona* sp. and should be useful for studying the diversity of other sponge-associated microorganisms.

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