Jurnal of Coastal Development
Volume 10, Number 1, October 2006 : 47 – 54
ISSN : 1410-5217
Accredited : 23a/Dikti/Kep/2004

PHYLOGENETIC DIVERSITY OF SECONDARY METABOLITE PRODUCING-BACTERIA ASSOCIATED WITH SPONGES FROM BANDENGAN WATERS, JEPARA

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Received : August, 4, 2006 : Accepted : September, 15, 2006

ABSTRACT

A total of 13 bacterial isolates associated with sponges 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. Six isolates were further selected based on the constructed dendrogram for subsequent DNA sequencings, resulted in the richness of secondary metabolite-producing bacteria associated with sponges from Bandengan having closest similarity to Pseudoalteromonas, Brachybacterium, Vibrio, alpha proteobacterium and uncultured bacterium clones.

Key words: Phylogenetic, diversity, secondary metabolite, bacteria, sponges

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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).

Sponges offer a rich source of unique and diverse secondary metabolites (Faulkner, 2000). Many of these compounds have potent pharmacological activities, including anti-tumor, antifungal, anti-viral, and anti-bacterial properties (Wallace, 1997). Several antibiotics have been isolated from marine sponges, e.g., plakortin from *Plakortis halichondroides*, and manoalide from *Luffariella variabilis* (De Silva and Scheuer 1980).

Serious obstacle to the ultimate development of most marine natural products that are currently undergoing evaluation and trials is the problem of supply due 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 anticipating the pressure

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

resulted from the search for bioactive compound from coral reef ecosystems, it is of importance to assess the application of ecological and biotechnological-based approaches on protecting the coral reef ecosystems. The results of the proposed work will further show alternative choice in order to protect the reefs from the search of bioactive compounds and to obtain the representatives of bioactive compounds from coral reef ecosystems.

MATERIALS AND METHODS

Sampling and isolation of sponge-associated bacteria

Colonies of sponges were collected from Bandengan water, Jepara, North Java, Indonesia by scuba diving from a depth of approximately 3 meters. 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 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).

Screening of coral bacteria with biological activity

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 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 30 µl of the coral bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 hours. 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 1R-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 ºC for 5 min; 30 cycles of denaturation (92 ºC for 1 min), annealing (50 ºC for 1,5 min), extension (68 ºC for 8 min); and final extension at 68 ºC for 10 min. Five µl aliquot PCR products were run using elektrophores on 6% acrilamide gel by using 1x TBE buffer.

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

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.

Tabel 1. Antibacterial Activity of Sponge Associated 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	-	+	-

Sign + : inhibited
Sign - : no inhibition

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

RESULTS AND DISCUSSION

Results

Inhibitory interaction test

The measurement of inhibition zone as indicator of the antibacterial potential of bacterial isolates associated with sponges from Bandengan waters is presented in the following Table 1.

Rapid grouping of active sponge bacteria

Based on the repetitive-PCR results and the constructed dendrogram of the isolates, 6 different isolates representing different groups (Fig. 1) were further selected for DNA sequencings.

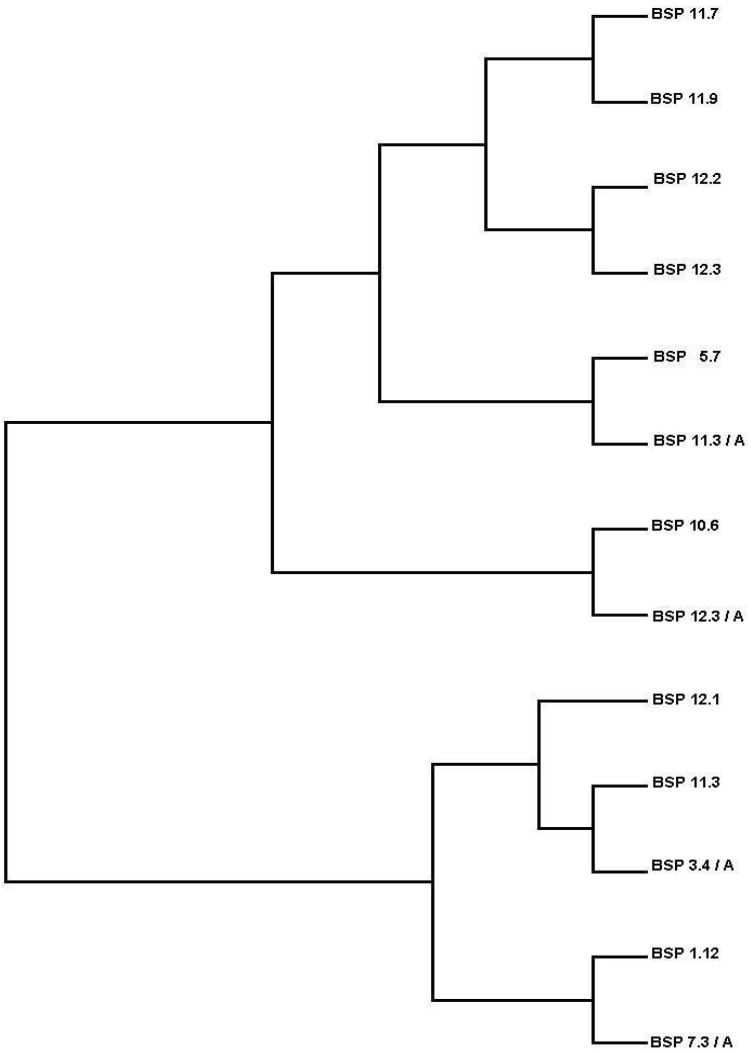

Fig 1. Dendrogram constructed based on rep-PCR of bacterial isolates from Bandengan, Jepara, North Java Sea

ISSN : 1410-5217
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Molecular identification

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

Table 2. 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

Discussion

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).

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).

Globally since 1995, there are signals of decreased interest in the search of new metabolites from traditional sources such as macroalgae, molluscs, tunicates and octocorals, and the number of annual reports on marine sponges stabilized. On the contrary, the metabolites from microorganisms is a rapidly growing field, due, at least in part, to the suspicion that a number of metabolites obtained from algae and invertebrates may be produced by associated microorganisms(Faulkner et al, 2000). Although it is still too early to define tendencies, it may be stated that the metabolites from microorganisms, are in most cases quite different from those produced by invertebrate hosts (Kelecom, 2002).

In the present study, we investigated the phylogenetic diversity of marine bacteria associated with sponges collected from Bandengan waters, North Java Sea. It is interesting to note that collected sponges offered potensial sources of secondary metabolite-producing bacteria as indicated by the results of actibacterial tests against indicator microorganisms.

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

Aeromonas hydrophila, *Staphylococcus aureus* and *Vibrio parahaemolyticus.*

The study has also revealed the power of using molecular based-aprroache i.e. repetitive-PCR (rep-PCR) as a mean of rapid grouping among sencondary metabolite-producing sponge bacteria, in which among 13 active isolates inhibited the growth of indicator microorganisms, 6 isolates were further selected for subsequent DNA sequencings. Radjasa et al (2007c), reported the application of rep-PCR for rapid grouping among marine psychrotrophic bacteria isolated from Makasar strait. The present results have confirmed the potential application of rep-PCR for rapid grouping of marine bacterial isolates including those asscociated with sponges.

Three genera *Pseudoalteromonas, Vibrio* and *Brachybacterium* were found to show antibacterial activities in the present study. 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).

Two isolates obtained BSP5.7 and BSP11.3 showed closest similarity to uncultured bacterium clones. It is not surprising that present work revealed the occurrence of isolates related to the unculturable part of microbial community associated with sponges, considering only about 1% of marine microorganisms have been successfully cultivated so far.

Sponge bacterium BS11.7 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).

CONCLUSION

In conclusion, the sponges collected from Bandengan waters, North Java Sea represent the potential sources of marine microorganisms associated with sponges with biological activity against bacterial pathogen *A. hydrophila, S. aureus* and *V. parahaemolyticus.*

The application of molecular based-technique, namely rep-PCR is a powerful method for rapid grouping a large number of marine isolates, especially those associated with sponges. This techique can be used to explore the richness of secondary metabolite producing-microorganims living in association with sponges.

ACKNOWLEDGEMENTS

This work was partly supported by the grant from Indonesian Ministry of Research and Technology within *Indonesian International Joint Research Grant* scheme (RUTI III) in the marine research area. The work was also part of a research grants provided by Lindbergh Foundation, USA and Indonesian Biodiversity Foundation (KEHATI) awarded to Ocky Karna Radjasa.

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

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

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