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GROWTH INHIBITION OF MEDICALLY ANTIBIOTIC RESISTANT BACTERIA BY SPONGE-ASSOCIATED BACTERIA

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

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ABSTRACT

The improper and uncontrolled uses of antibiotics against pathogenic bacteria have resulted in the occurrence of Multi Drugs Resistant bacteria. There is now an urgency to find alternative antibiotics to combat these bacteria. The metabolites from microorganisms are 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. Therefore, there is a shift in the search for secondary metabolites from terrestrial to marine environment. Sponge-associated microorganisms are among of the most interesting and promising marine natural product sources, which produce polyketide and non ribosomal peptide products with various biological activities. In this study, marine bacteria were isolated from sponge Haliclona sp. *collected from* North Java Sea, *and were screened for antibacterial activity against MDR strains. One out of 32 bacterial isolates were successfully screened and were found to be active against MDR strains, strain* Escherichia coli *and strain* Proteus sp., *respectively. These active isolates were also capable of amplifying NRPS gene fragments necessary for the biosynthesis of non ribosomal peptides. The identification results revealed that the active isolates are* Arthrobacter sp.

Key words: *antibacterial, marine bacteria, sponge Haliclona sp., MDR*

***Correspondence** : Phone +62-24-7460038; Fax +62-24-7460039; e-mail: ocky_radjasa@yahoo.com

INTRODUCTION

Marine organisms are well known to have specific relationships with numerous microorganisms, and sponges are no exception to this. The studies of these associations underscore the importance of marine sponges as living fossils as well as their significance in drug discovery. The present state of our knowledge on sponge-bacteria associations is based mainly on the culture of associated microorganisms from the sponges (Thakur and Muller, 2005).

The biology of the bacterium-sponge relationship has elicited considerable interest among researchers investigating marine organisms as sources of natural products. Antimicrobial compounds have been isolated from sponge-associated bacteria on numerous occasions, and this has prompted

the suggestion that microbial symbionts play a role in the defense of their host sponge. Marine sponges produce a wide array of other natural products and bioactive secondary metabolites (Faulkner, 2000).

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.

Advanced techniques of molecular biology such as Polymerase Chain Reaction (PCR), in particular the application of degenerated primers of Non-ribosomal peptide synthetases (NRPS) to amplify gene fragments from peptide producers has allowed screening on the presence of non ribosomal peptides among secondary metabolite-producing microorganisms (Marahiel *et al.*, 1997; Radjasa *et al,* 2007a).

In this work, we reported the potential of marine bacteria associated with sponge *Aaptos* sp. for the production of secondary metabolites against Multi Drugs Resistant (MDR) bacterial strains coupled with PCR based-screening for the presence of non-ribosomal polypeptide synthetases.

MATERIALS AND METHODS

Sampling and isolation of sponge-associated bacteria

Colonies of sponge were collected from the vicinity of Panjang island, Jepara, North Java Sea, Indonesia by scuba diving. Upon collection sponge colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA. The tissues were then rinsed with sterile seawater and homogenized with blender. The homogenized 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).

Antibacterial test

Antibacterial test of sponge-associated bacteria against MDR bacteria was performed by using an overlay method. Multi Drugs Resistant (MDR) bacteria (*Pseudomonas* sp., *Escherichia coli*, *Proteus* sp. *Enterobacter* sp. and *Staphylococcus* sp. used in this study were obtained from Laboratory of Clinical Microbiology, Kariadi Hospital, Semarang). Culture of each MDR bacterium in the logarithmic phase (ca. 10^9 cells ml^{-1}) was mixed with TSB soft agar medium (1% v/v), which were then poured on to the respective agar surface previously inoculated with sponge-associated bacteria and incubated for 4 d. The plates were then incubated at room temperature for 48 hours. Antibacterial activity was defined by the formation of inhibition zones around the bacterial colonies.

PCR-based screening of NRPS producing bacterial strains

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). 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´)(Radjasa *et al.*, 2007a,b).

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NRPS-PCR was performed with a thermal cycler (Eppendorf Inc, Germany) as follows: 1 µl template DNA, and 1 µl of each of the appropriate primers, which were then put into puReTaq Ready-To-Go PCR beads (Amersham Biosciences Europe GmbH, Germany). 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.

Phylogenetic analysis.

A phylogenetic tree was constructed using maximum-likelihood analysis. Only sequences of type strains were included in tree calculation. 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. Phylogenetic analysis was performed with

RESULTS AND DISCUSSION

Results

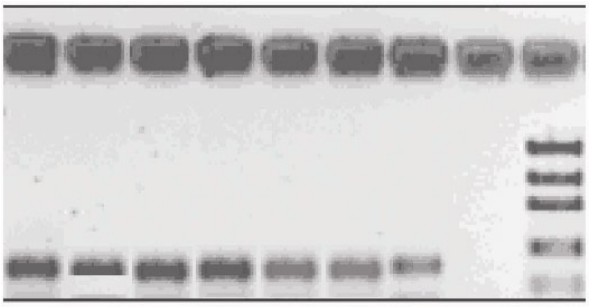
Out of 32 sponge isolates one isolate was found to inhibit the growth of 2 Multi Drugs Resistant (MDR) strains **(Table 1).**

Table 1. Antimicrobial activity of sponge bacterium SFNB.3 against MDR strains

No	MDR Strain	Inhibition zone (mm)
1	*Escherichia coli*	9.9
2	*Proteus* sp	11.2

Further screening for the presence of gene fragments of Non-ribosomal Peptide Synthetase (NRPS) that this active isolate was capable of amplifying the NRPS gene fragments **(Fig 1.).**

Molecular identification of the active sponge isolates based on 16S rDNA, revealed that the active strain is belonging to the members of *Arthrobacter* **(Table 2).**



SFNB3 + M

Fig. 1. PCR amplification of NRPS gene fragments; + control *Pseudomonas fluorescens* DSM No. 50117; M is DNA markers

Table 2. Identification of active bacterial strain associated with *Haliclona* sp.

No	Bacterial isolate	Closest relative	Homology (%)	Accession number
1	SFNB.3	*Arthrobacter nicotianae*	98	EU402968

A. phylogenetic tree showing the appropriate of affiliation of the active strain SFNB.3 is shown at **fig.2**

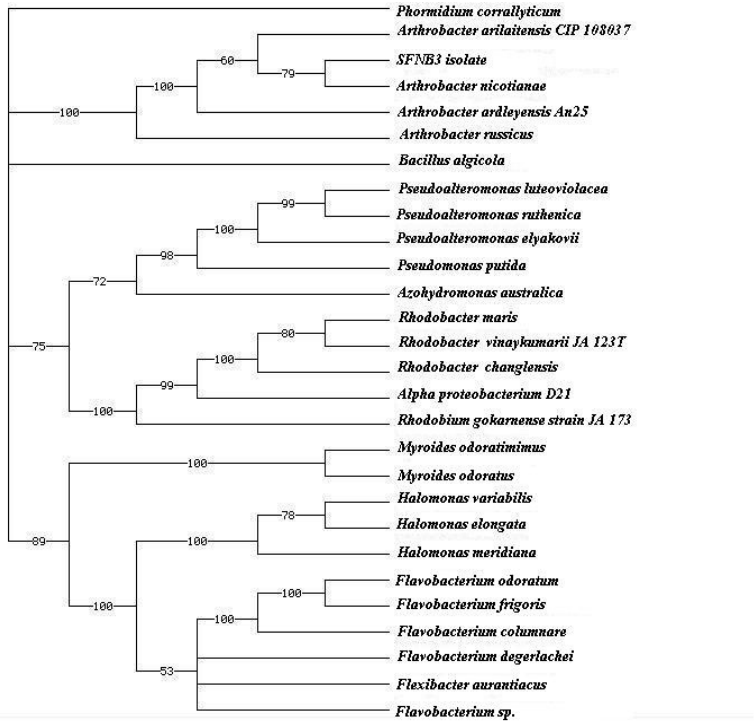


Fig 2. *A. Pgylogenetic* tree of the active sponge bacterium SFNB.

DISCUSSION

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, 2007b).

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.

There has been an increasing concern regarding the collecting reef's organisms for

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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 (Sukarmi and Radjasa, 2007).

The present study indicated that a marine bacterium associated with sponge *Haliclona* sp. showed strong growth inhibition against MDR strains **(Table 1).** This offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria in particular among the member of Multi Drugs Resistant (MDR) strains.

In this study one isolate, SFNB.3 showing closest relative to *Arthrobacter nicotianae* **(Table 2)** inhibited the growth of *A. coli* and *Proteus* sp. **(Table 1).** A number of bacteria with antimicrobial activities from the marine sponges *Aplysina aerophoba* and *Aplysina cavernicola* was reported. The sponge isolates were affiliated with the low (*Bacillus*) and high G + C Gram-positive bacteria (*Arthobacter*, *Micrococcus*), as well as the α-Proteobacteria (unknown isolate) and γ-Proteobacteria (*Vibrio*, *Pseudoalteromonas*) (Henstchel *et al,* 2001). The sponge isolates show antimicrobial activities against Gram-positive and Gram-negative reference strains but not against the fungus *Candida albicans.* A sponge belonging to *Aplysina* includes heterogeneous bacteria *Bacillus* sp., *Micsrococcus* sp., *Arthrobacter* sp., *Vibrio* sp., and *Pseudoalteromonas* sp.(Lee *et al*, 2001).

CONCLUSSION

In conclusion, sponge *Haliclona* sp. exhibited secondary metabolite producing-marine bacteria with antibacterial potential against MDR strains and therefore futher study is needed to isolate and to elucidate the responsible compounds.

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