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GROWTH INHIBITION OF CLINICALLY RESISTANT BACTERIA BY MARINE BACTERIA ASSOCIATED WITH SPONGE *Aaptos* sp.

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ABSTRACT

The improper and uncontrolled uses of antibiotics against pathogenic bacteria have resulted in the occurrence of Multi Drugs Resistant (MDR) strains. There is now an urgency to find alternative antibiotics to combat the MDR strains. 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 Aaptos sp. collected from North Java Sea, and were screened for antibacterial activity against MDR strains. Three out of 64 bacterial isolates were successfully screened and were found to be active against MDR strains, in which 2 isolates (SPA1 and SPA5) were active against resistant strain Escherichia coli and 1 isolate (SPA21) against resistant 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 Halomonas aquamarina, Alpha proteobacterium, and Pseudoalteromonas luteviolacea

Keywords: *grwoth inhibition, marine bacteria, sponge Aaptos sp., resistant bacteria*

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INTRODUCTION

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).

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antimicrobial resistance, which become a major health problem world wide (Goldmann and Huskins, 1997). In the context of Indonesian population, a recent work by Kuntaman et al (2005) confirmed the occurrence of Fluoroquinolone-resistant *Escherichia coli* based on population survey of 3.996 persons. There is now an urgency more than ever to find alternative antibiotics to combat the Multi Drugs Resistant (MDR) strains.

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 in particular Multi Drugs Resistant (MDR) strains.

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 (**Fig.1**) by scuba diving. Upon collection sponge colonies were put into sterile

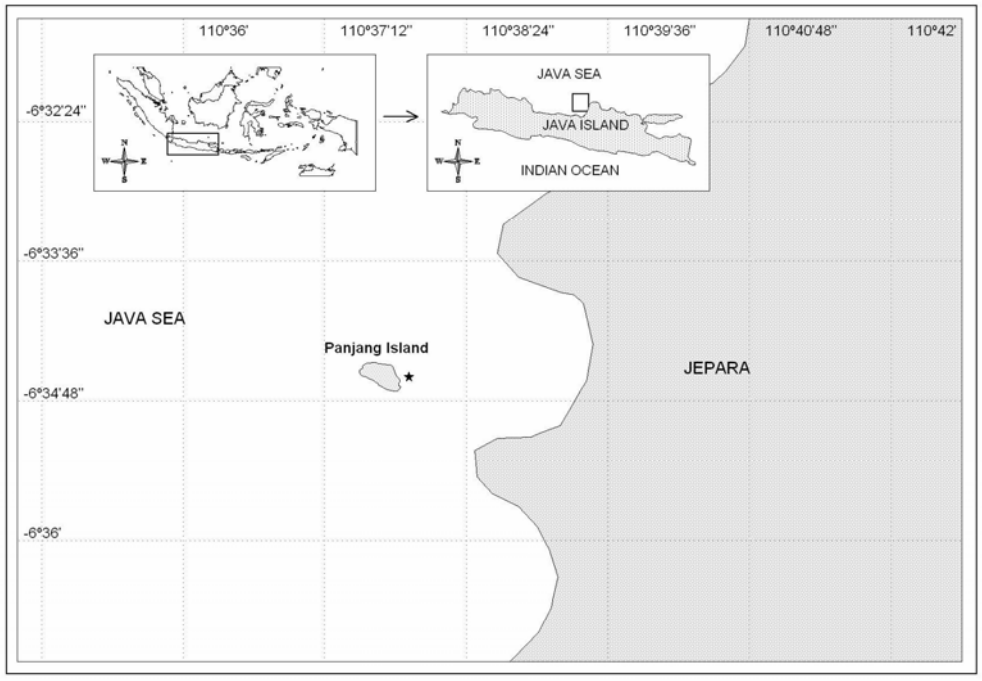

Fig 1. Sampling site at Panjang island, Jepara, North Java, Indonesia

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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).

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.

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

Results

Out of 64 sponge isolates tested, three isolates were found to inhibit the growth of 3 Multi Drugs Resistant (MDR) strains **(Table 1).**

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Table 1. Antimicrobial activity of sponge bacteria against MDR strains

No	Sponge Isolate	MDR Strain	Inhibition zone (mm)
1	SPA1	*Escherichia coli*	9.3
2	SPA3	*Escherichia coli*	15.0
3	SPA21	*Proteus* sp.	8.6

Further screening for the presence of gene fragments of Non-ribosomal Peptide Synthetase (NRPS) among the active isolates showed that all active strains were capable of amplifying the NRPS gene fragments (**Fig 2**.)

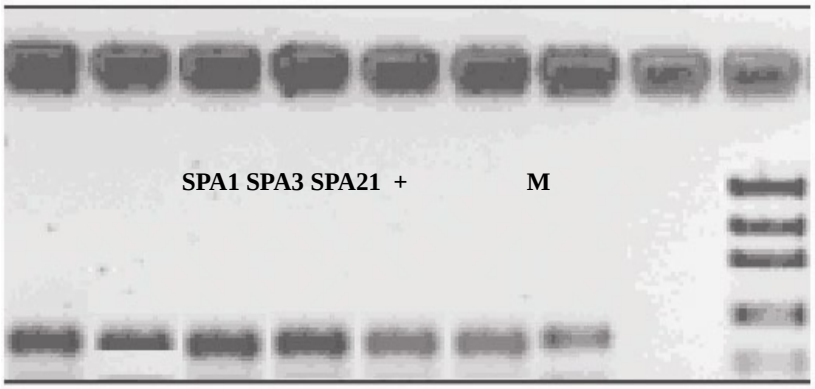

Fig. 2. PCR amplification of NRPS gene fragments; + control *Pseudomonas fluorescens* DSM No. 50117; M is DNA markers

Molecular identification of the active sponge isolates based on 16S rDNA showed that the active strains are belonging to the members of *Halomonas*, *Pseudoalteromonas* and *Alpha Proteobacterium* **(Table 2).**

Table 2. Molecular identification of active bacterial strains associated with *Aaptos* sp.

No	Bacterial isolate	Closest relative	Homology (%)	Accession number
1	SPA1	*Halomonas aquamarina*	99	DQ372908
2	SPA2	*α-proteobacterium* D21	100	DQ399723
3	SPA21	*Pseudoalteromonas luteoviolacea*	100	DQ504310

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, b).

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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 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 marine bacteria associated with sponge *Aaptos* sp. showed strong growth inhibition indicator microorganisms (Table 1). It is believed that the emergence of Multi Drugs Resistants (MDR) bacteria is correlated with improper uses of antimicrobial agents such as many prescriptions not taken correctly, antibiotics sold without medical supervision, and spread of resistant microbes due to the lack of hygiene. Considering the prevalence of antimicrobial resistance has emerged in Indonesia, the present study offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria especially to handle the occurrence of Multi Drugs Resistant (MDR) strains.

In this study one isolate, SPA1 showing closest relative to *Halomonas aquamarine* (Table 2) inhibited the growth of *A. coli* but not *Proteus* sp. (Table 1). Very limited information is available on the antibacterial activity of the member of genus *Halomonas.* Very recently, a work reported by Bitzer et al (2006) regarding the production of antibiotic Aminophenoxazinones produced by *Halomonas* sp. isolated from water sample of the East Frisian Wadden Sea.

On the other hand, sponge bacterium SPA21 revealed the highest homology (100%) to the bacterium *Pseudoalteromonas luteoviolacea*. This bacterium inhibited the growth of MDR strain *Proteus* sp. 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. Interestingly, this coral bacterium TAB4.2 also amplified NRPS gene fragments, and following DNA cloning and sequencing, a 279 bp long DNA fragment was obtained and the deduced amino acid sequence showed conserved signature regions for peptide synthetases and revealed a high similarity to NosD (40% identity), a multifunctional peptide synthetase from *Nostoc* sp. GSV224, and NdaB (44% identity), a peptide synthetase module of *Nodularia spumigena* (Radjasa et al, 2007a). Species of *Pseudoalteromonas* have also been isolated from tunicates (Holmstrom, 1998) and sponges (Ivanova et al, 2002).

Sponge bacterium SPA3 showed high similarity to α-proteobacterium D21. Radjasa et al (2007c) isolated bacterium from sponge *Haliclona* sp. collected from Bandengan waters, Jepara, North Java Sea, which was closely related to α-proteobacterium Z143-1 (98%), a bacterium isolated from Philippine tunicate that produce anti-*Staphylococcus aureus* metabolite heptylprodigiosin (de Guzman, unpublished). This result confirmed the presence of α-proteobacterium which was found to be dominantly associated with sponge *Rhopaloides odorabile* from

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geographically different areas and, as specific symbiont of sponges. Furthermore, Webster and Hill (2001) reported that an α-proteobacterium as found to be associated with sponges *Theonella swinhoei, Aplysina aerohaba,* and *Haliclona panacea.*

The present study also strongly revealed the ecological rationale for sponge *Aaptos* sp. and its associated microorganisms for the maintenance of antimicrobial defenses. Seawaters typically contains 10^7 viruses, 10^6 bacteria, 10^3 fungi, and 10^3 microalgae/ml (Engel et al, 2002), including those which have been identified as causative agents in marine infectious diseases (Correa, 1997). Given that marine invertebrates and their symbionts are continuosly exposed to a broad array of potentially deleterious microorganisms, it is reasonable that the production of bioactive secondary metabolites could act as fundamental mechanism of antimicrobial defense.

In conclusion, sponge *Aaptos* sp. exhibited secondary metabolite producing-marine bacteria with antibacterial potential against MDR strains. Further works are needed to clarify the antibacterial compounds responsible for the growth inhibition toward MDR strains.

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