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Antibacterial Activity of Marine Bacterium *Pseudomonas* sp. Associated with Soft Coral *Sinularia polydactyla* against *Streptococcus equi* Subsp. *zooepidemicus*

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Abstract: A marine bacterium associated with soft coral *Sinularia polydactyla* collected from Bandengan water, Jepara, North Java Sea, Indonesia, was successfully screened for antibacterial activity against pathogenic bacterium *Streptococcus equi* subsp. *zooepidemicus* K6.72 isolated from infected monkey of the island of Bali and identified based on morphological, biochemical and molecular methods. Marine bacterium was identified as *Pseudomonas* sp. based on its 16S rDNA and was found to amplify gene fragments of Non-ribosomal peptide synthetase (NRPS). Cloning and subsequent sequencing, a 360 bp long DNA fragment was obtained and the deduced amino acid sequence showed conserved signature regions for peptide synthetases and revealed a high similarity of 61.1% to genes peptide synthetase of *Bacillus subtilis*.

Key words: Screening, soft coral-associated bacteria, secondary metabolites

INTRODUCTION

Soft corals are an important and diverse group of colonial invertebrates belonging to the Phylum Coelenterata (Cnidaria), Class Anthozoa, Subclass Octocorallia. One of the major groups, the order Alcyonacea consists of hundreds of different species including the member of *Sinularia* can dominate many Indo-Pacific reefs (de Nys *et al*., 1991). Furthermore, one of the reasons for the evolutionary success of the alcyonacean soft corals in the Indo-Pacific is considered to be the high level of secondary metabolites commonly found in their tissues (Sammarco and Coll, 1992). Thus, it has been the main reason for the searching of secondary metabolites with various biological activities from softcorals.

Among streptococcus, *Streptococcus equi* subsp. *zooepidemicus* has been known as the cause of infection of a wide variety of animals such as pigs, cows, goats and monkeys (Salasia *et al*., 2004), which resulted in the occurrence of pneumonia, meningitis and arthritis. Infections of streptococcus group C (SGC) have been reported from Mexico (Edwards *et al*., 1988) and the island of Bali, Indonesia (Salasia *et al*., 2004). Further, Bradley *et al*. (1991), reported that infection of SGC was caused by pathogenic *Streptococcus zooepidemicus* (72.7%).

Bioactive-producing marine invertebrates, including softcorals are insufficient for producing commercial quantities of metabolites of interest. Therefore, a solution to overcome the problem of supply is needed. It has been widely 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*., 2007). Thus, it is important to highlight the possible role of marine bacteria associated with soft coral in providing solution to the problem of infection by pathogenic bacterium *Streptococcus equi* subsp. *epidemicus*. Bacteria-soft coral association that occurs on the soft coral surface then could be of great interest to search for potential use as new source of antibiotics.

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

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from peptide producers has allowed screening on the presence of non ribosomal peptides among secondary metabolite-producing microorgamisms (Marahiel *et al*., 1997; Radjasa and Sabdono, 2003; Radjasa *et al*., 2007).

In this study, we reported the potential of marine bacterium associated with soft coral *Sinularia polydactyla* for the production of secondary metabolites against pathogenic *Streptococcus equi* subsp. *zooepidemicus* K6.72 bacterium coupled with PCR based-screening for the presence of non-ribosomal polypeptide synthetases.

MATERIALS AND METHODS

Sampling and isolation of soft coral-associated bacteria: Colonies of soft coral were collected from Bandengan water, Jepara, North Java, Indonesia by scuba diving from a depth of 5 m. Upon collection soft coral colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA) and brought to Marine Station, Diponegoro University. 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 h. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al*., 2000).

Isolation of *Streptococcus equi* subsp. *zooepidemicus* bacterium: Isolation was carried out at Faculty of Veterinary Medicine, Gajah Mada University, Yogyakarta, Indonesia. Bacterium K6.72 was isolated from infected monkey from the area of natural tourism forest of Alas Kedaton, Bali island. Blood Agar Plate (blood agar base (40 g L^{-1}) which was then mixed with 7% defibrinated blood from goat) was used to grow the isolate.

Morphological and biochemical characterization of isolate K6.72: Gram and biochemical tests (glucose, lactose, manitol, alanine, arabinose, rafinose, maltose, arginine, sorbitol, inuline and hemolysis activity) were carried out according to a method previously described (Salasia *et al*., 2004). The reference strain *Streptococcus equi* subsp. *zooepidemicus* was obtained from Institut für Pharmacologie und Toxicologie, Justus-Liebig-Universität Gießen, Gießen, Germany.

Screening of soft 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, 5 bacterial isolates were tested against isolate K6.72. One hundred microliter culture of 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. Biological activity was defined by the formation of inhibition zones around the paper disk. Isolate showed biological activity against K6.72 was chosen for further screening based on PCR technique by using specific primers of Non-ribosomal peptide synthetase (NRPS).

Antibacterial test: Inhibitory interaction test of isolate TASC.16 against pathogenic *Streptococcus equi* subsp. *zooepidemicus* K6.72 was performed by using the agar disk-diffusion method. One hundred microliter culture of target microorganism in the logarithmic phase (ca. 10^9 cells mL^{-1}) was spread on to agar medium. Three paper disks (8 mm; Advantec, Toyo Roshi, Ltd, Japan) containing 30 μL of the softcoral 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*. (2007) by the formation of inhibition zones greater than 9 mm around the paper disk.

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). Non-ribosomal peptide sythetases (NRPS) primers were prepared in the Marine Microbiology, IFM-GEOMAR, Kiel, Germany. Amplification of peptide synthetase gene fragments was carried out with the NRPS degenerated primers as described by Radjasa *et al*. (2007).

PCR amplification and sequencing of 16S rRNA gene fragments: PCR amplification of partial 16S rRNA gene of marine bacterium TASC.16 and monkey isolate K6.72, purification of PCR products and subsequent sequencing analysis were performed according to the methods of Thiel and Imhoff (2003) and Salasia *et al*. (2001), respectively. The determined DNA sequences of strains were then compared for homology to the BLAST database.

Cloning and sequencing of (putative) peptide synthetase domain: 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).

RESULTS

Screening of softcoral bacteria with biological activity: Screening among 5 marine bacteria associated with softcoral *Sinularia polydactyla* by using test organism revealed that only one isolate, TASC.16 capable of inhibiting the growth of *Streptococcus equi* subsp. *zooepidemicus*, while the rest of isolates showed no activity.

Inhibitory interaction test: The measurement of inhibition zone as indicator of the antibacterial potential of isolate TASC.16 against pathogenic K6.72 is presented in the following Table 1.

PCR-based screening: As shown in the Fig. 1, isolate TASC.16 was capable of amplifying the gene fragments of Non-ribosomal peptide synthetases (NRPS) indicated by the presence of single band having similar height to the positive control of *Pseudomonas fluorescens* DSM 50117.

Microbiological characterization: The microbiological characterization (morphological and biochemical tests) is presented in the Table 2.

Molecular identification: A comparison of the 16S rRNA gene sequence of isolate TASC.16 with sequences from GenBank demonstrated that this strain is most closely related with *Pseudomonas* sp. (accession number EF111238) with a homology of 98%.

On the other hand, the pathogenic isolate K6.73 was found to be closely related to *Streptococcus equi* subsp, *zooepidemicus* isolate 122 (Alber *et al*., 2005) with a homology of 98% (Acession number AJ715480).

Putative peptide synthetase sequence: Following cloning and subsequent sequencing, a 360 bp long DNA fragment was obtained and the deduced amino acid sequence showed conserved signature region for peptide synthetases and revealed a high similarity to genes of peptide synthetase of *Bacillus subtilis* (Table 3).

Table 1: Antibacterial activity of softcoral isolate TASC.16 against pathogenic K6.72

	Inhibition zone (mm)			
Isolate	I	II	III	Average
TASC.16	15.50	16.10	15.70	15.76

Table 2: Microbiological characterization of isolate K6.72

No.	Type of test	Result	Percentage
1	Gram	+	-
2	Shape	Coccus	-
3	Hemolysis activity	β	-
4	Glucose	+	100
5	Lactose	+	90
6	Sorbitol	+	100
7	Inuline	-	0
8	Manitol	-	0
9	Arabinose	-	0
10	Rafinose	-	0
11	Alanine	-	0
12	Arginine	+	100
13	Eskuline	-	0

Sign +: positive result, Sign -: negative result

Table 3: Characterization of NRPS gene fragments of bacterium TASC16

Strain	Length (bp)	Closest gene fragment	Similarity (%)	Acc. number
TASC.16	360	Peptide synthetase genes of *Bacillus subtilis*	61.1	Z34883

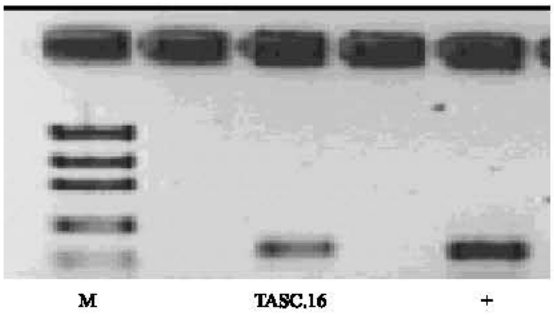

Fig. 1: PCR amplification of NRPS gene fragments; + control *Pseudomonas fluorescens* DSM No. 50117; M is DNA markers

DISCUSSION

An attempt was carried out to estimate the potential of marine bacteria associated with soft coral *Sinularia polydactyla* as the source of antibacterial compounds in particular against the pathogenic *Streptococcus equi* subsp. *epidemicus*.

Both microbiological and molecular-based methods confirmed that pathogenic bacterium K6.72 is *Streptococcus equi* subsp. *zooepidemicus*. This result is very similar to the occurrence of this bacterium during streptococcal outbreak in Bali island in 1994 (Salasia *et al*., 2004) that infected pigs and monkeys from the same area of Alas Kedaton, Bali. *Streptococcus equi* subsp. *zooepidemicus* is well known as an important pathogen of

the horse being associated with respiratory tract infections of foals and with uterine infections in mares (Salasia, 2001). In addition this bacterium can be isolated from infections of a wide variety of animals, including pigs, sheep, cows, goats, foxes, birds, rabbits, guinea pigs and monkeys. All of these animals might be potential reservoirs for infections of humans.

It is interesting to note that softcoral bacterium *Pseudomonas* sp. TASC.16 showed strong growth inhibition against *Streptococcus equi* subsp. *zooepidemicus* K6.72. This raises the possibility the use of softcoral bacteria as the source of antibacterial compounds for controlling the pathogenic bacterium *Streptococcus equi* subsp. *zooepidemicus*. The members of genus Pseudomonas are well known to produce various peptide natural products including antibacterial (Mossialos *et al*., 2002), biosurfactants (Morikawa *et al*., 1993; Amada *et al*., 2000; Roongsang *et al*., 2003) and antifungals (Ramette *et al*., 2001; Nielsen *et al*., 2002; Sorensen *et al*., 2002).

Bacterium *Pseudomonas* sp. TASC.16 has also amplified the gene fragments of Non-ribosomal peptide sythetases (NRPS) that showed homology to the genes of peptide synthetases of *Bacillus subtilis*. The members of genus Bacillus were reported to produce also peptide natural products. Cereulide, the emetic toxin was putatively a product of non-ribosomal peptide synthesis of *Bacillus cereus* (Toh *et al*., 2004) which is known as the causative agent of two distinct types of toxin-mediated food-borne illness, diarrhoel and emetic syndromes (Drobniewski, 1993). Furthermore, other Bacillus species, such as *B. brevis*, *B. subtilis* and *B. licheniformis*, produce cyclic peptides using non-ribosomal peptide synthesis (Stachelhaus *et al*., 1995).

In conclusion, softcoral bacterium *Pseudomonas* sp. TASC.16 capable of producing antibacterial compound as demonstrated by antibacterial test against pathogenic *Streptococcus equi* subsp *zooepidemicus* K6.72. It contained the NRPS gene fragment as shown by PCR screening. Further works are needed to clarify the responsible compounds in controlling the pathogenic strain tested.

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