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Original paper

SCREENING OF SECONDARY METABOLITE-PRODUCING BACTERIA ASSOCIATED WITH CORALS USING 16S rDNA-BASED APPROACH

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ABSTRACT

A total of 42 coral-associated bacteria were screened for their ability to produce secondary metabolites by using 16S rDNA-based approach. A specific oligonucleotides primer was used to amplify DNA non-ribosomal peptide synthetase (NRPS). Five strains were found to carry the NRPS gene and successfully inhibit the growth of other coral-associated and pathogenic bacteria.

The phylogenetic characterization revealed that four strains belonged to currently known species Pseudoalteromonas luteoviolacea, Vibrio tubiashi, Vibrio carchiariae and Bacillus iodinum. On the other hand, one strain was apparently closely related with an unknown group of the gamma Proteobacteria, which was only recognized as uncultured clone 141H2.

Key words: Screening, coral-associated bacteria, secondary metabolites, 16S rDNA

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INTRODUCTION

Marine organisms in particular marine invertebrates from coral reef ecosystem have become sources of great interest to natural product chemistry, since they produce metabolites with different biological activities. Marine invertebrates, which are plentiful in the Indo-Pacific regions, are rich in secondary metabolites and are becoming targets of continuing search for antibacterial agents. However, the problem of supply has hampered the development of these secondary metabolites produced by marine invertebrates. Thus, finding an alternative sources is a great prominence.

Since 1995, there are signals of decreased interest in the search of new metabolites from traditional sources such as macroalgae, molluscs, tunicates, softcorals 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 (Fenical,

1993; Pietra, 1997; Bernan *et al*, 1997; Faulkner *et al*, 2000; Jensen and Fenical, 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).

Serious obstacle to the ultimate development of most marine natural products that are currently undergoing evaluation and trials is the problem of supply. 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 addition, it has often been proven extremely difficult, and some cases impossible, to provide from invertebrates or macroalgae 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. Thus, new ways have to be found.

It has been clearly established that microbial cells attached firmly to almost any surface submerged in marine environments. The cells grow, reproduce, and produce extracellular polymers that provide structure to the assemblage termed as biofilm. Paul *et al* (1986); Coffroth (1990) and Kim (1994) mentioned that it is not surprise mucus-covered coral surfaces are often covered by micro-organisms mostly undestructive.

In relation to bacteria associated with corals, particularly their interaction among coral surface colonizers, it is reasonable to expect that the indigenous microbial population may be adapted to a competitive conditions, at least in part, due to the competition on the available nutrients. Thus, one possible mechanism for these bacteria to survive is by producing secondary metabolites that enhance their existence.

Studies regarding screening on secondary metabolites-producing coral-associated bacteria are important for understanding principal processes of inhibitory interaction among coral-associated bacteria as well as their biotechnological potentials.

It is also expected that there are still a number of unexplored culturable coral-associated microorganisms in the reef environments. Such information might be desirable, as some of these bacteria may serve beneficial purposes as a source of secondary metabolites including novel natural products such as antibiotics, lipids, pigments, and pharmaceuticals.

The 16S rDNA-based approaches have become a standard for studying the phylogeny and diversity of marine microorganisms (Bowman *et al*, 1997; DeLong *et al*, 1997; Nogi *et al*, 1998; Radjasa *et al*, 2001a, 2001b; Urakawa *et al*, 1999a, 1999b). In addition, 16S rDNA-based approach has been very successful in the search of secondary metabolites in particular by using specific degenerated primers (Konz and Marahiel, 1999; Seow, 1997).

In this context, it is important to assess the application of PCR-based approach on screening of coral-associated microbial populations with specific consideration of the secondary metabolites-producing parts which have been up to now strongly neglected in comparison to the invertebrate parts.

In an effort devoted to the search of bioactive compounds synthesized by marine-invertebrate-associated bacteria, we studied bacterial strains collected at the surfaces of the corals harvested at Teluk Awur, on the north coast of Jepara, Central Java for their possible development as an alternative source of secondary metabolites from marine microorganisms.

MATERIALS AND METHODS

Sampling and isolation of coral-associated bacteria

The corals representing different life-forms (massive, submassive, branching and foliose) were collected from Teluk Awur water, North Java Sea by scuba diving. Upon collection coral was put into sterile plastic bags (Whirl-Pak, Nasco, USA) and brought to Marine Station, Department of Marine Science, Diponegoro University, Jepara, Central Java, Indonesia. The coral was immediately rinsed with sterile seawater and scrapped with sterile knife. The resultant tissues were then serially diluted, and the dilution was spread on ½ strength ZoBell 2216E marine agar medium (Radjasa *et al*, 2001a) and incubated at room temperature 2 x 24 hours. On the basis of morphological features, colonies were randomly picked and purified by streaking method.

DNA extraction

Strains were cultured at 20^{o}C on 3 ml ½ ZoBell 2216E broth medium. After harvesting, cells were washed and suspended in sterile distilled water and 40 µl of the suspension were mixed with 10 µl proteinase K (1 mg ml^{-1}) (Sigma Chemical Co., St. Louis, MO, USA) and 50 µl of 2xK buffer (40 mM Tris buffer, 0.2% Nonidet P-40, 0.2 mM EDTA, 1% Tween 20, distilled water, pH 8.0). The mixture was heat-treated at 60 ^{o}C for 20 min, followed by 100 ^{o}C for 10 min, then cooled rapidly on ice for 10 min and centrifuged at 8000 rpm for 5 min. The resulting lysate was removed and directly used for PCR without further purification.

PCR amplification

PCR amplification was carried out according to the method of Radjasa et al (2001). Universal primers described by Weisburg et al (1991) was used for PCR amplification. For screening purpose, a specific oligonucleotide primer of non-ribosomal polypeptide synthetase (Konz and Mahariel, 1999) was used for PCR amplification. PCR amplification was conducted in a thermal cycler (Mini Cycler TM, MJ Research Inc, Watertown, MA, USA). Amplified DNAs were examined by horizontal electrophoresis on 1% agarose gel in TAE electrophoresis buffer (40mM Tris, 20 mM acetate, 2 nM EDTA) with 1 µl aliquots of PCR product. The gel image was taken using a densitograph imaging analyzer (ATTO, Tokyo, Japan).

DNA sequencing

PCR products were purified with spin columns (Amicon**,** Inc, Beverly, MA, USA) and were prepared using a Sequi Therm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) for subsequent sequencing with an automated DNA sequencer (Pharmacia LKB Biotech, Uppsala, Sweden). The determined DNA sequence was then compared for homology with BLAST search.

Inhibitory interaction tests

Inhibitory interaction tests among degenerated primers-amplified bacteria were performed by using diffusion agar method (Conception *et al*, 1994; Radjasa *et al*, 1999). One hundred µl culture of each target microorganism in the logaritmic phase was spread on to agar medium using glass spreader. Several paper discs were placed in the agar surface and filled with 10 µl of each positive primers-carrying bacterial strain. The plates were then incubated at room temperature for 2 x 24 hours. The inhibitory interaction was determined by the appearance of inhibition

zones around the paper disc and was measured quantitatively.

RESULTS AND DISCUSSION

Polyketides and non-ribosomal peptides represent large families of secondary metabolites and numerous natural products belonging to these groups are widely used as pharmaceuticals, industrial agents or agrochemicals (Silakowski *et al*, 2000). Both types are biosynthesized by extremely large polyfunctional enzyme systems within the protein. The responsible biosynthetic proteins are known as polyketide synthases (PKS) and non-ribosomal polypeptide sythetases (NRPS) (Cane, 1997).

PCR-based screening allowed a rapid evaluation of many isolates among coral-associated bacteria produced secondary metabolites, particularly the member of non-ribosomal polypeptide synthetases (NRPS). As indicated in the **Figure 1**, 5 strains out 42 bacterial strains, positively carried NRPS gene as represented by the occurrence of DNA bands on the agarose gels.

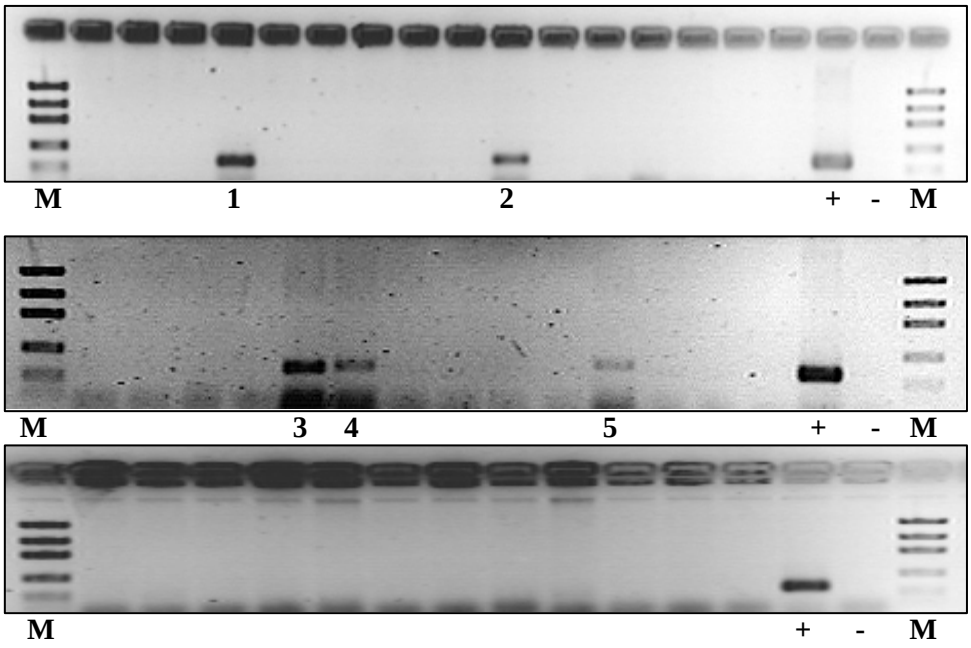

Fig 1. PCR-based screening of secondary metabolite-producing strains 1 (TAB4.2); 2 (TAB4.3); 3 (TAB2.1); 4 (TAF2.4a); 5 (TAF4.1); + control (*Pseudomonas fluorescens* DSM No. 50117); M: DNA markers.

Positive result was obtained in 5 strains from 42 strains, namely 2 strains in the first; 3 strains in the second agarose gels, and no strain was found in the third agarose gel. All positively produced bands were in accordance with the DNA size of the control strain *Pseudoalteromonas fluorescens.*

Not all proteins are synthesized on the ribosome, and small polypeptides can be assembled by peptide synthethases just as other compounds. Most non-ribosomal

peptides from microorganisms are classified as secondary metabolites, which rarely have a role in primary metabolism, growth or reproduction, instead have evolved to somehow benefit the producing organisms (Neilan *et al*, 1999). The products of microbial non-ribosomal peptide synthesis include the immuno-suppressant cyclosporine and antibiotics such as gramicin S, tyrocin A and surfactins (Von Dohren and Kleinkauf, 1996).

Non-ribosomal peptide synthesis is achieved by the thiotemplate function of modular enzyme complexes known collectively as peptide synthetases. It has been established that the specific combination of modules and various functional domains within the peptide synthetase determines the structure and the activity of peptide product (Neilan et al, 1999).

To estimate the ecological role of positive strains as well as their biotechnological potency, inhibitory tests were carried out against other coral-associated bacteria and pathogenic bacteria. As described in **Table 1**, NRPS strains were capable of inhibiting the growth both coral-associated and pathogenic bacteria.

Chemical ecology is the study of chemically mediated interaction between organisms, and between organisms and their environment (Gerhart, 1985) which has also been extended into marine environment. The growth inhibition of coral-associated bacteria by NRPS strains showed an important role of secondary metabolites in the ecology of coral-associated bacteria perhaps in term of competition for space and nutrients between species. It could be also considered that the occurrence of secondary metabolites within NRPS strains is a possible reason for their successful existence in the surface of corals.

Table 1. Inhibition interaction of NRPS carrying-strains against coral-associated and pathogenic bacteria.

NRPS strain	Coral bacteria					Pathogenic bacteria				
	Hal	Pho	Sal	Oce	Sil	Col	Aur	Har	Par	Ang
TAB.4.2	+	+	+	+	+	+	+	+	+	+
TAB4.3	+	+	+	+	+	+	+	+	+	+
TAB2.1	+	+	+	+	+	+	+	+	+	+
TAF2.4a	+	+	+	+	+	+	+	+	+	+
TAF4.1	+	+	+	+	+	+	+	+	+	+

Hal: *Halomonas salina*; **Pho**: *Photobacterium* sp HAR7; **Sal**: *Salinicoccus roseus*; **Oce**: *Oceanobacillus iheyensis*; **Sil***: Silicibacter* sp ; **Col**: *Escherichia coli*; **Aur**: *Staphylococcus aureus*; **Har**: *Vibrio harveyi*; **Par**: *Vibrio parahaemolyticus*; **Ang**: *Vibrio anguilarum*; + (inhibited)

On the other hand, the capability of NRPS strains indicates their potential development in controlling several pathogenic bacteria. A work is now being undertaken to isolate and purify the bioactive compounds produced by strain TAB4.2 in collaboration with Department of Chemistry, McMaster University, Canada.

To estimate genetic affiliation among NRPS carrying strains, all 5 NRPS strains were sequenced. The phylogenetic characterization of each strain is summarized in **Table 2**. Strain TAB4.2 was mostly related to *Pseudoalteromonas luteoviolacea* with a sequence similarity of

98% and was assigned to the genus *Pseduoalteromonas*. The sequence of TAB4.3 was most similar to that of *Vibrio carchiariae*. Best conformity of strain TAB2.1 with reported sequence from cultured microorganisms was found with other member of the genus *Vibrio, V. tubiashi*. In contrast, strain TAF2.4a showed high homology to *Bacillus iodinum* (98%). In this regards, the 16S rDNA sequencing helped resolve the exact taxonomic position of NRPS strains.

Table 2. Phylogenetic characterization of NRPS strains among coral-associated bacteria.

No	Strain	Closest relatives	Homology (%)	Acess. no
1	TAB4.2	*Pseudoalteromonas luteoviolacea*	98	X82144
2	TAB4.3	*Vibrio carchiariae*	97	X74693
3	TAB2.1	*Vibrio tubiashi*	97	X74725
4	TAF2.4a	*Bacillus iodinum*	98	X83813
5	TAF4.1	*Uncultured Clone 1401H2*	97	AF442075

On the contrary, strain TAF4.1 showed a high level of similarity of 97% only with unidentified clone 1401H2. Considering the level of similarity with uncultured microorganism, and the fact that high similarity was found only with unidentified clone, thus, the isolate confirms its existence in the natural environment as well as a novelty of secondary metabolite-producing strain. Further studies are needed to clarify the detail taxonomic characteristic of strain TAF4.1

CONCLUSION

The present study indicates that using 16S rDNA-based approach it is possible to determine not only the diversity of coral-associated bacteria, but also an application of non-ribosomal peptide synthetases (NRPS) primers to increase the screening efficiency of secondary metabolite-producing strains.

The inhibitory results supported the efficiency of the application of PCR technique using specific NRPS primers in which all NRPS strains inhibited the growth of test strains.

The 16S rDNA sequence analysis revealed that NRPS strains among coral-associated bacteria belonged to bacterial genera *Pseduoalteromonas, Bacillus, Vibrio* and one strain, TAF4.1 was closely affiliated with *uncultured clone 1401H2*.

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