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Ecological Role of A Softcoral-Associated Bacterium *Arthrobacter* sp. on Marine Biofilm-Forming Bacteria

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A marine bacterium species associated with softcoral *Sinularia* sp. collected from vicinity of Peucang island, Ujung Kulon, West Java, was successfully screened for estimating its ecological role through inhibiting marine biofilm-forming bacteria isolated from the surrounding colonies of *Sinularia* sp. and was identified as closely related to *Arthrobacter nicotianae* based on its 16S rDNA structure. The bacterium was found to inhibit the growth of four biofilm-forming isolates (*Vibrio harveyi, V. fortis, Staphylococcus sciuri,* and *Tenacibaculum marilutum*) indicating the significance of secondary metabolite production which may provide important defensive functions against fouling microorganisms. The isolate was capable of amplifying gene fragments of non-ribosomal peptide synthetases. A 416 bp long DNA fragment was obtained and the deduced amino acid sequence showed conserved signature regions for the peptide synthetases and revealed a high similarity to that of *Actinoplanes teichomyceticus* (62.5% identity).

Key words: ecology, softcoral, bacteria, biofilm

Softcorals 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 *Sinularia* which can dominate many Indo-Pacific reefs (Coll and Sammarco 1986). Furthermore, one of the reasons for the evolutionary success of the alcyonacean softcorals in the Indo-Pacific is considered to be the high level of secondary metabolites which are commonly found in their tissues (Sammarco and Coll 1992).

One of the most interesting aspects of the softcorals is that fouling organisms usually do not colonize their surfaces and it is believed that antifouling represents another ecological role of secondary metabolites in the Alcyonacea (Sammarco and Coll 1992). In general, secondary metabolites are thought to enhance the fitness of the producing species (Engel *et al*. 2002).

Marine biofouling, is a natural process resulting from organism growth on underwater surfaces which causes huge economic losses to marine industries. In seawater, the microbial population on surfaces produce primary biofilm, which is generally thought to be a prerequisite for the attachment and metamorphosis of fouling organisms (Callow and Callow 2002).

It has been widely reported that many biologically active natural products from marine invertebrates have striking similarities to metabolites of their associated microorganisms including bacteria (Proksch *et al*. 2002; Thiel and Imhoff 2003). Thus, it is important to highlight the possible ecological role of marine bacteria associated with softcoral in providing protection of the host from fouling microrganisms. Bacterial-softcoral association that occurs on the softcoral surface then could be of great interest to search for potential use as commercial antifouling compounds.

Non-ribosomal peptides represent one of the large families of secondary metabolites and numerous natural products belonging to these groups. They are widely used as pharmaceuticals, industrial agents or agrochemicals. This type of peptide is sythesized by extremely large polyfunctional enzyme systems within the protein known as non-ribosomal polypeptide sythetases (NRPS) (Silakowski *et al*. 2000).

Advanced techniques of molecular biology such as the Polymerase Chain Reaction (PCR), in particular the application of degenerated primers of NRPS to amplify gene fragments from peptide producers, have allowed the screening for the presence of non-ribosomal peptides among secondary metabolite-producing microorganisms (Marahiel *et al.* 1997; Ayuso-Sacido and Genilloud 2004; Radjasa *et al*. 2007a,b).

In this work, we report the ecological relevance role of marine bacteria associated with softcoral *Sinularia* sp. for the production of secondary metabolites which are antagonistic toward marine biofilm-forming bacteria. This is coupled with PCR based-screening for the presence of non-ribosomal polypeptide synthetases.

MATERIALS AND METHODS

Sampling and Isolation of Softcoral-Associated Bacteria. Colonies of soft coral *Sinularia* sp. were collected from the vicinity of Peucang island, Ujung Kulon, West Java, Indonesia by scuba diving. Upon collection softcoral colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA. The tissues were then rinsed with sterile seawater (filtered by using 0.2 μm membrane filter and autoclaved) and scraped off with a sterile knife. The resultant tissues were serially diluted, spread on 50% strength ZoBell 2216E marine agar medium and incubated at room temperature for

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48 hours. On the basis of morphological features, colonies were randomly picked, and purified by making streak on plates (Madigan *et al*. 2000).

Isolation of Marine Biofilm-Forming Bacteria. Bacterial isolation was carried out by using a method modified from Harder *et al*. (2003). Four pre-sterilized glass slides had been deployed in 4 different directions around softcoral colony for a week. The biofilm developed in these glass slides were then collected and put into sterile petri dishes, rinsed with sterile seawater and scrapped off with a sterile knife. The resultant mixture was diluted. A 100 µl aliquot of each dilution was spread onto 50% strength ZoBell 2216E and incubated at room temperature for 48 hours. Colonies with distinguishing feature were selected and purified.

Inhibitory Interaction Test. The ability of softcoral-associated bacteria to inhibit the growth of marine biofilm-forming bacteria was performed by using an overlay test method. Aliquots culture of each marine biofilm-forming bacterium in the logarithmic growth 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 softcoral-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´) designed from conserved regions of adenylation domains of various bacterial peptide synthetase sequences (GenBank accession numbers: AAK81824, AAK81827, AAK81826, AAC82549, CAA40561, CAC48362, CAA11796, CAC48369, CAC48369, AAF42473, BAB69322, CAB38518, AAG02364, AAG02355, AAG02356, CAA67248, CAB93684, CAB93684, CAB93683, AAC68816, AAC44129, CAA65394, AAG05812, AAG05789, AAG05789, AAF40220, AAD51026, CAC11137, AAB96629). The sequence of the reverse primer was based on the signature sequence of the superfamily of adenylate forming enzymes TSGXTGXPK (motif A3) found in peptide synthetases, but also in acetyl-CoA synthetases. The sequence of the forward primer, based on the motif KAGGAY(LV)P (motif A2), is highly conserved for peptide synthetases which are involved in non ribosomal peptide synthesis (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). Each 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. PCR amplification of partial 16S rRNA genes of active strains and marine biofilm-forming 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 (Sabdono and Radjasa 2008).

Cloning and Sequencing of the 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).

Phylogenetic Analysis. A phylogenetic tree was constructed using maximum-likelihood analysis. Alignment positions with < 50% of sequences of the entire set of data having 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 the PAUP software package.

RESULTS

Softcoral isolate, SFNB.5 was found to effectively inhibit the growth of 4 marine biofim-forming bacteria. This was identified as shown Table 1. Molecular identification indicated that SFNB.5 showed highest similarity to the member of genus *Athrobacter* (Table 2).

Identification of biofilm-forming bacteria is presented in the Table 3. This indicates that these bacteria belonged to the members of *Vibrio*, *Staphylococcus,* and *Tenacibaculum.* PCR-based screening by using specific primers NRPS revealed that bacterial isolate SFNB.5 was capable of amplifying the gene fragments of NRPS as shown in the Fig 1. To investigate the genetic potential of isolate SFNB.5 to produce secondary metabolites, a 416 bp long DNA fragment was obtained. The deduced amino acid sequence did indeed show conserved signature regions for peptide synthetases (Table 4).

Table 1 Inhibitory interaction of marine biofilm-forming bacteria by softcoral bacterial isolate SFNB.5

Isolate	Biofilm-forming bacteria			
	BFB1.7	BFB1.8	BFB2.1	BFB2.6
SFNB.5	+	+	+	+

Table 2 Molecular identification of softcoral bacterium SFNB.5

Isolate	Closest relative	Similarity (%)	Acc. Number
SFNB.5	*Arthrobacter nicotianae*	97	AJ315492

Table 3 Molecular identification of marine biofilm-forming bacteria

Isolate	Closest relative	Similarity (%)	Acc. Number
BFB2.1	*Vibrio fortis*	99	AJ514916
BFB1.8	*Staphylococcus sciuri*	97	S83569
BFB1.7	*Vibrio harveyi*	97	AJ672389
BFB2.6	*Tenacibaculum marilutum*	97	AY661693

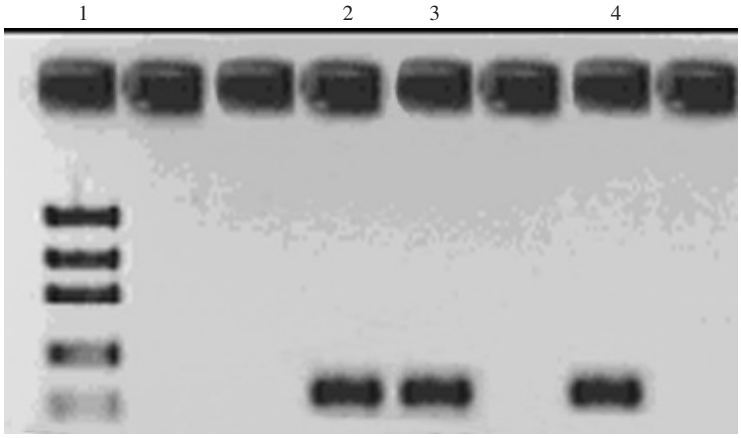

Fig 1 PCR amplification of NRPS gene fragments. Lane 1, DNA markers; Lane 2-3, SFNB.5; Lane 4, control *Pseudomonas* DSM 50117.

Table 4 Characterization of NRPS gene fragments

Strain	Closest gene fragment	Similarity (%)	Acc. Number
SFNB.5	*Actinoplanes teichomyceticus glycopeptide* gene	62.5	X56928

A comparison of the 16S rRNA gene sequence of strain SFNB.5 with sequences from GenBank demonstrated that this isolate is affiliated to the genus *Arthrobacter*. The phylogenetic tree shown in Fig 2 shows that isolate SFNB.5 is most closely related with *A. nicotianae* with a similarity of 97%.

DISCUSSION

Within minutes of immersing a clean surface in seawater, it adsorbs a molecular conditioning film, consisting of dissolved organic material. Bacteria colonize this within hours, along with unicellular algae and cyanobacteria. These early colonizers form a biofilm, an assemblage of attached cells sometimes referred to as microfouling. A macrofouling community may then develop and overgrow the initial microfouling (Callow and Callow 2002).

Accumulating evidence that invertebrate-associated microbes contribute to the production of bioactive compounds has prompted the search for antimicrobial compounds from these microoorganisms (Webster *et al.* 2001; Proksch *et al.* 2002). Growth inhibition of marine biofilm-forming bacteria by isolate *Arthrobacter* SFNB.5 demonstrates the so far uncharacterized secondary metabolites of this isolate lead to antagonistic activity and hence they may lead to advantages in the competition for space colonization of the softcoral surface. This assumption is supported by the fact that the NRPS positive strain SFNB.5, exhibited antibacterial activity against four marine biofilm-forming bacteria. The efficient inhibition of marine biofilm-forming bacteria by strain SFNB.5 may be beneficial not only for the respective bacterium but also for the softcoral host since it further protects the softcoral from fouling microorganisms.

It is interesting to note that the active softcoral isolate SFNB.5, which is closely related to *Arthrobacter* species, is indeed very distantly related to the member of marine biofilm-bacteria (*Vibrio, Staphylococcus,* and *Tenacibaculum*) isolated from the surrounding soft coral colony, despite the fact that both were exposed to the same ecological conditions. Wahl (1989) reported that biotic surfaces frequently harbor species-specific microbial communities that can be highly variable and distinct from those found in the surrounding environment. Furthermore, Engel *et al*. (2002) reported that there is evidence that there are symbiotic microbes which can chemically defend the host microbial colonization.

The members of the genus *Arthrobacter* has been known to produce various chemicals such as acetylchloline having pharmacological significance (Mohapatra and Bapuji 1998); cold-active antimicrobial compounds (O'Brien *et al*. 2004) and enzymes (Fujita *et al*. 1990).

Most non-ribosomal peptides from microorganisms are classified as secondary metabolites, since they rarely play a role in primary metabolism, such as growth or reproduction but have evolved to somehow benefit the producing organisms (Neilan *et al*. 1999). Products of the microbial non-ribosomal peptide synthesis include the immunosuppressant cyclosporine and common antibiotics such as gramicin S, tyrocin A and surfactins (Kleinkauf and von Doehren 1996).

The comparison of the derived amino acid sequence of the putative non ribosomal peptide synthetase of strain SFNB.5 revealed a high similarity to sequence fragments of known glycopeptide synthetases of *Actinoplanes teichomyceticus*. The highest similarity was found with sequences of organisms belonging to the Actinomycetes, from which many genera possess non-ribosomal peptide synthetase genes (Jensen *et al.* 2005). *A. teichomyceticus* produced the glycopeptide teicoplanin, which is used for the treatment of serious infections caused by Gram-positive pathogens (Borghi *et al*. 1989; Sosio *et al.* 2004)

In conclusion, the softcoral bacterium SFNB.5 exhibited secondary metabolites which inhibit the growth of marine biofilm-forming bacteria. The present study highlights the ecological role of secondary metabolite producers amongst colonizers of softcoral *Sinularia* sp.

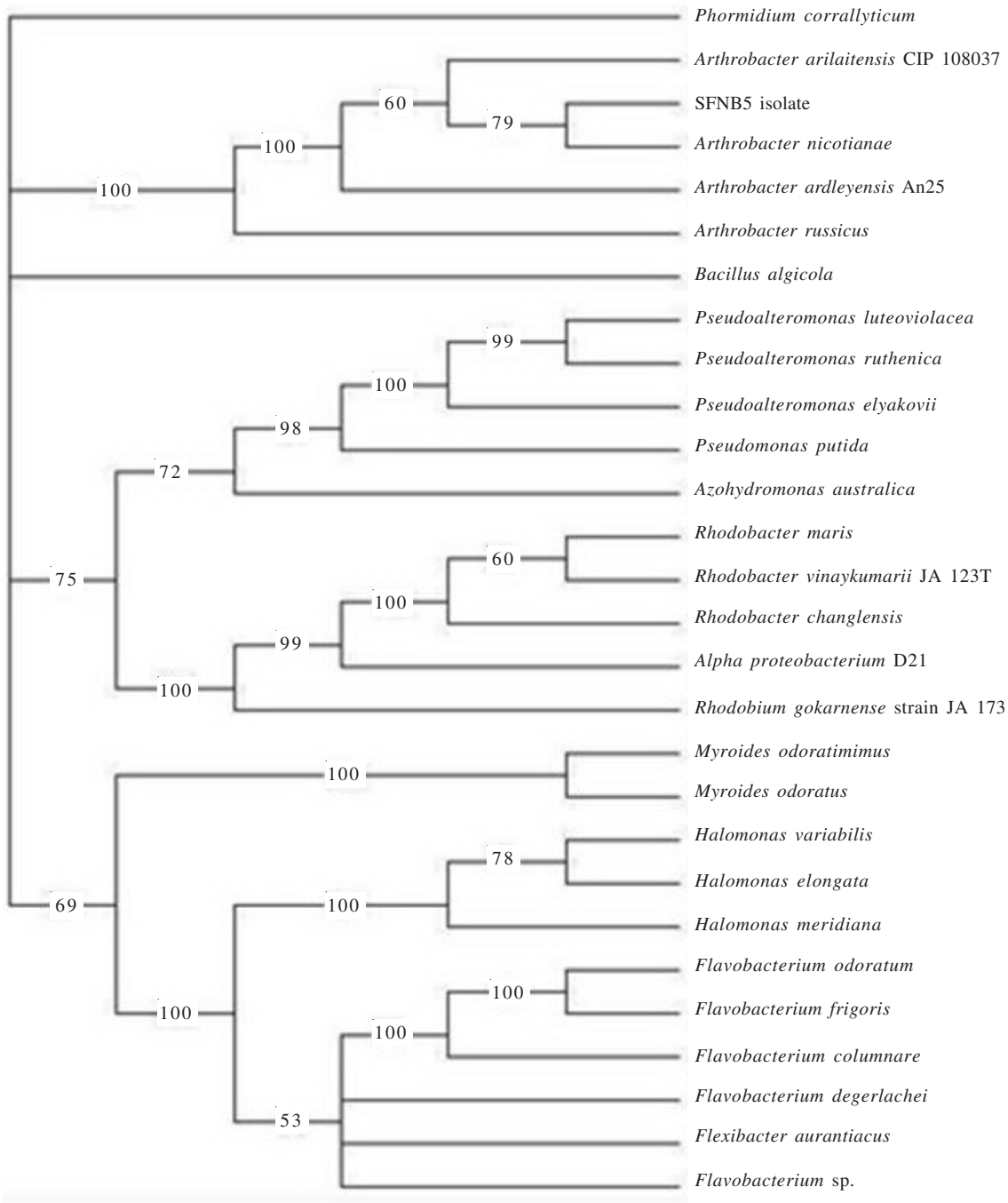

Fig 2 Phylogenetic affiliation of soft coral isolate SFNB.5.

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