

ANTIFOULING ACTIVITY OF BACTERIA ASSOCIATED WITH SOFT CORAL *Sarcophyton* sp. AGAINST MARINE BIOFILM-FORMING BACTERIA

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ABSTRACT

Marine bacteria associated with soft coral *Sarcophyton* sp collected from vicinity of Peucang island, Ujung Kulon, West Java, were successfully screened for antifouling activity against marine biofilm-forming bacteria isolated from the surrounding colonies of *Sarcophyton* sp. Six bacterial isolates were found to inhibit the growth of at least one of 7 biofilm-forming isolates. The most active strain USP3.37 was identified as *Pelagiobacter variabilis* by using 16S rDNA gene sequence analysis. Similarly, the active strains USP3.3, USP8.43, USP3.12, USP3.16 and USP8.6 were identified as *Arthrobacter nicotianae*, *Shewanella* alga, *Pseudomonas synxantha*, *Pseudomonas falgida*, *Pseudovibrio denitrificans* and *Bacillus aquamaris*, respectively. USP3.37 strain was found to amplify gene fragments of non-ribosomal peptide synthetase (NRPS). This raises the possibility the use of soft coral bacteria as the source of antibacterial compounds for controlling the antifouling in the sea. Therefore, this bacterium would be better to select eco-friendly antifouling compounds than the other antibacterial activities.

Keywords: *Pelagiobacter variabilis*, biofilm, antifouling, 16S rDNA,

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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 group, order Alcyonaria consists of hundreds of different species including the genus of *Sarcophyton*. This leather coral, *Sarcophyton* sp., is easily recognized by its mushroom shape. The thick, fleshy stalk attaches the colony to the bottom, and feathery, 8-branched polyps extend from the broad, disc-like cap (Benayahu et al., 2002). 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).

Marine biofouling, despite a natural process as a result of organism growth on underwater surfaces (Pereira et al, 2002), 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).

Bacteria growing on the surfaces of marine algae and other organisms live in a highly competitive environment in which space and access to nutrients are limited (Burgess et al., 1999; Slattery et al, 2001). Previous studies have shown that a high percentage of marine epibiotic bacteria produce antimicrobial metabolites compared with the number of planktonic isolates that produce such metabolites (Boyd et al. 1999a; Boyd et al., 1999b; Jensen and Fenical, 1994; Lemos et al., 1986). One of the most interesting aspects of the soft corals is that fouling organisms usually do not colonize their surfaces and it is believed that antifouling represents another ecological role of secondary metabolite in the alcyonacean (Sammarco and Coll, 1990).

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; Imhoff and Stöhr, 2003). Thus, it is important to highlight the possible role of marine bacteria associated with soft coral in providing an alternative to the commercial metal-based antifouling coatings that are believed to be an environmental hazards due to their toxicity. Bacteria-soft coral association that occurs on the soft coral surface then could be of great interest to search for potential use as commercial antifoulants.

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 presense of non ribosomal peptides among secondary metabolite-producing microorganisms (Marahiel *et al.*, 1997).

In this work, we reported the potential of marine bacteria associated with soft coral *Sarcophyton* sp for the production of secondary metabolites against marine

biofilm-forming bacteria 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 *Sarcophyton* sp. were collected from the vicinity of Peucang island, Ujung Kulon, West Java, Indonesia (Figure 1) by scuba diving. Upon collection soft coral colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA. The tissues were then rinsed with sterile seawater and scrapped 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 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan et al, 2000).

Isolation of marine biofilm-forming bacteria

Isolation was carried a method modified from Harder et al (2003). Four pre-sterilized glass slides had been deployed in 4 different around soft coral colony for a week. The biofilm developed in these glass slides were then put into sterile petri dish, rinsed with sterile seawater and scrapped off with a sterile knife. The resultant mixture were diluted. One hundred µl of each dilution was spreaded onto ½ strength ZoBell 2216E and incubated at room temperature for 48 hours. Colonies with distinguished feature were selected and purified.

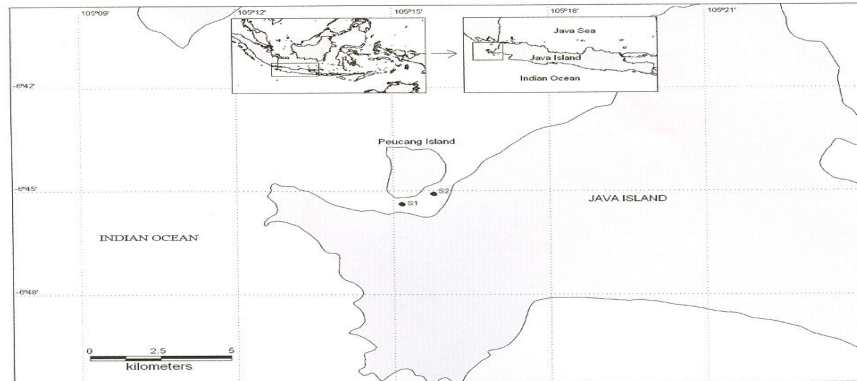
Antifouling test

Antifouling tes of soft coral-associated bacteria against marine biofilm-forming

bacteria was performed by using an overlay method. Culture of each marine biofilm-forming 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

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

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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 ($-80^{\circ}C$) and thaw ($95^{\circ}C$). 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')(Marahiel *et al.*, 1997).

NRPS-PCR was performed with a thermal cycler (Eppendorf Inc, Germany) as follows: 1 μ l template DNA, and 1 μ l 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 $95^{\circ}C$, annealing for one minute at $70^{\circ}C$ and extension for two minutes at $72^{\circ}C$, respectively.

PCR amplification and sequencing of 16S rRNA gene fragments.

PCR amplification of partial 16S rRNA gene of active strains, purification of PCR products and subsequent sequencing analysis were performed according to the method of Thiel and Imhoff (2003). The determined DNA sequences of strains were then compared for homology to the BLAST database.

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 the PAUP software package (Swofford, 1988).

RESULTS AND DISCUSSION

Antifouling activity of bacteria associated with soft coral

Screening among 98 marine bacteria associated with soft coral *Sarcophyton sp.* by using test organisms revealed that only six isolates are capable of inhibiting the growth of biofilm-forming bacteria, while the rest of isolates showed no activity (Table 1 and Figure 2). USP3.37 isolate was the most active strain that inhibited 6 of 7 bacterial tested.

Table 1. Screening result of antifouling activity of soft coral associated bacteria

Strain	Biofilm-forming Bacteria						
	BFB1.1	BFB2.8	BFB1.7	BFB1.8	BFB1.6	BFB2.1	BFB2.6
USP3.3	-	+	+	+	+	-	+
USP3.37	-	+	+	+	+	+	+
USP8.43	+	+	-	-	+	-	-
USP3.12	-	+	-	-	-	-	-
USP3.16	-	+	+	+	+	+	-
USP8.6	+	+	-	-	-	-	-



Fig 2. Screening of antifouling

Molecular identification and Phylogenetic analysis

A comparison of the 16S rRNA gene sequence of active strains with sequences from GenBank demonstrated that the most active strain USP3.37 was identified as

Pelagibacter variabilis with a homology of 96%. Similarly, the other active strains USP3.3, USP8.43, USP3.12, USP3.16 and USP8.6 were identified as *Arthrobacter nicotianae*, *Shewanella alga*, *Pseudomonas synxantha*, *Pseudovibrio denitrificans* and *Bacillus aquamaris*, respectively (Table 2). Phylogenetic tree of those active strains was constructed by using PAUP 4.0 version (Figure 3).

Table 2. Molecular identification of active strains

No	Strain	Closest relative	Homology (%)
1	USP3.16	<i>Pseudovibrio denitrificans</i>	97
2	USP3.3	<i>Arthrobacter nicotianae</i>	97
3	USP8.43	<i>Shewanella alga</i>	97
4	USP3.12	<i>Pseudomonas synxantha</i>	98
5	USP3.37	<i>Pelagiobacter variabilis</i>	96
6	USP8.6	<i>Bacillus aquamaris</i>	99

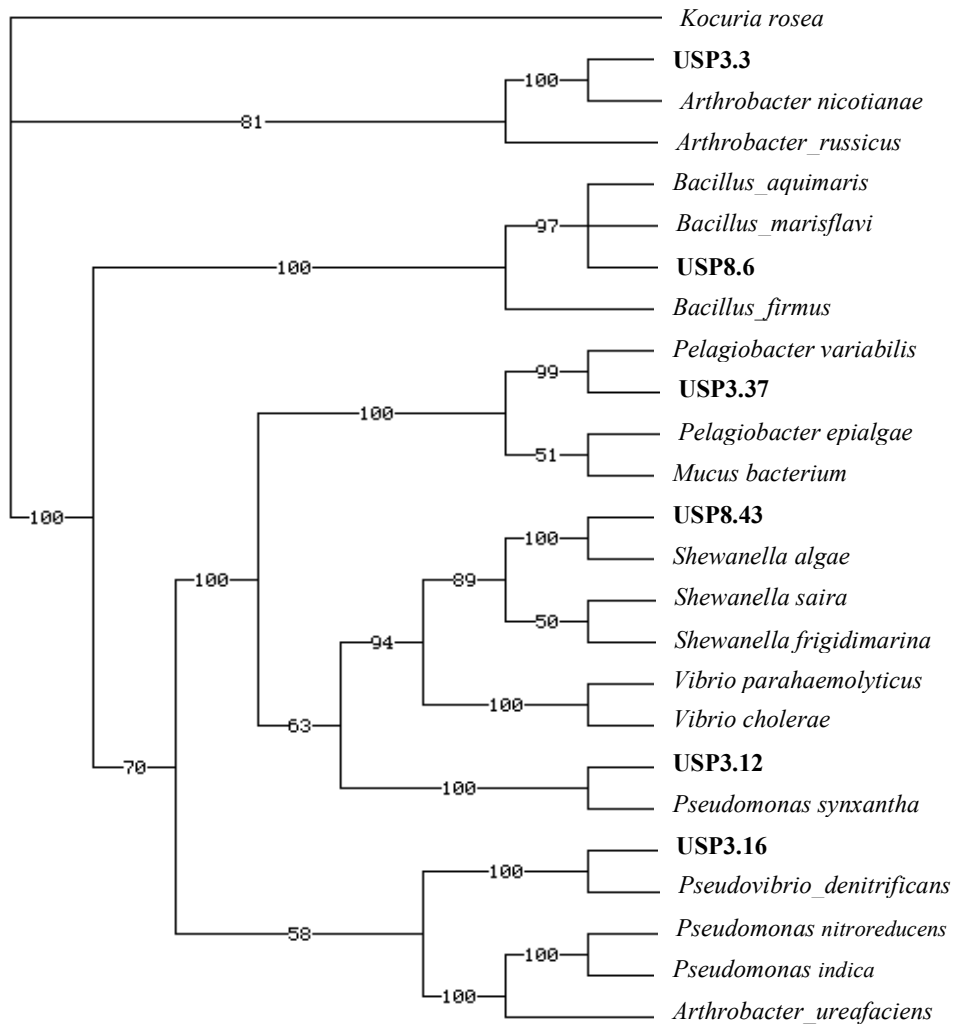


Fig 3. Phylogenetic tree based on comparative 16S rRNA gene sequence analysis of *Phelagiobacter* species showing the phylogenetic affiliation of strain USP3.37. *Kocuria rosea* was used as *outgroup*. The bar indicates 2% sequence divergence.

PCR-based screening

As shown in the figure 4, isolate USP3.37 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.

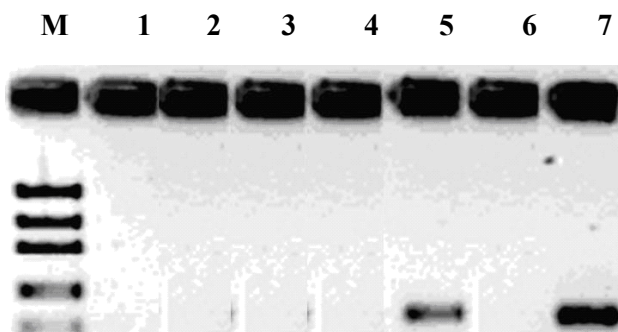


Fig 4. PCR amplification of NRPS gene fragments; M: DNA marker; 1: USP3.16; 2: USP3.3; 3: USP8.43; 4: USP3.12; 5: USP3.37; 6: USP8.6; 7: + control *Pseudomonas fluorescens* DSM No. 50117

DISCUSSION

An attempt was carried out to estimate the potential of marine bacteria associated with soft coral *Sarcophyton sp* as the source of antibacterial compounds in particular against biofilm-forming bacteria.

Inhibitory interactions among coral-associated bacteria that occur on the coral surface are of great interest to search for secondary metabolite-producing bacteria. Isolation and screening for secondary metabolite-producing bacteria in coral reef ecosystems have been strongly neglected until now. Our results highlight one softcoral-associated bacterium (USP3.37) carrying the NRPS gene. This bacterium is 96% identical to *Pelagiobacter variabilis* based on its 16S rRNA gene sequence.

Growth inhibition of biofilm-forming bacteria by NRPS strain USP3.37 (Table 1) demonstrates the so far uncharacterized

secondary metabolites of strain USP3.37 lead to antagonistic activity and, may hence

lead to advantages in the competition for space and nutrients with other coral-associated bacteria. The efficient inhibition of biofilm-forming bacteria strain BFB1.1, BFB2.8, BFB1.7, BFB1.8, BFB1.6, BFB2.1 and BFB2.6 by strain USP3.37 may further reflect the potential role of coral bacteria in controlling biofouling on coral surface.

Not all proteins are synthesized on ribosomes, and small polypeptides can be assembled by peptide synthetases just as other compounds. Most non-ribosomal peptides from microorganisms are classified as secondary metabolites. 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). Interestingly, the organism closest related to USP3.37, *Pelagiobacter variabilis*, owns a non-ribosomal peptide synthetase,

which produces the *anticancer antibiotics pelagiomicins* (Mearns-Spragg et al, 1988; Yan et al., 2002; Imamura et al., 1997). Although the biological function of the gene product remains unknown, the feasibility that the respective gene detected in strain USP3.37 codes for a non ribosomal peptide synthetase is high.

The present work highlights the production of secondary metabolites by a symbiotic coral bacterium (UPS3.37) carrying the NRPS gene. The expression of the NRPS gene accounts for the biosynthesis of various natural products with different biological activity (Silakowski et al, 2000). Hence, the application of molecular approach through PCR using specific NRPS primers provides rapid detection and is suitable to greatly improve the screening efficiency for secondary metabolite-producer among coral-associated bacteria against biofilm-forming bacteria.

In conclusion, softcoral bacterium *Pelagiobacter variabilis* USP3.37 capable of producing antifouling compound as demonstrated by antibacterial test against biofilm-forming bacteria. It contained the NRPS gene fragment as shown by PCR screening. Further works are needed to clarify the responsible compounds in controlling the microfouling strain tested. This raises the possibility the use of softcoral bacteria as the source of antibacterial compounds for controlling the antifouling in the sea.

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