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CORALS AS SOURCE OF BACTERIA WITH ANTIMICROBIAL ACTIVITY

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ABSTRACT

In this study we examined marine bacteria associated with different corals (Porites lutea, Galaxea fascicularis, Acropora sp. and Pavona sp.) *collected from vicinity of Panjang island, Jepara, North Java Sea, Indonesia for their antimicrobial activities against the* bacteria Echerichia coli, Bacillus subtilis, Staphylococcus lentus and the yeast Candida glabrata. *A total of 13 bacterial isolates belonged to the members of* Bacillus, Vibrio, Micrococcus, Pseudoalteromonas, Arthrobacter *and* Pseudovibrio *were found to inhibit the growth of at least one test strain. Further examinations among the biologically active strains by using PCR with specific primers of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) resulted in the presence of NRPS gene fragments in the 2 members of* Bacillus *and* Micrococcus *and PKS gene fragments in the 2 members of* Bacillus *and* Vibrio. *Following cloning and sequencing of the PCR products, the fragments from* Bacillus *BM1.5 and* Micrococcus *BJB showed sequence identity with peptide synthetase genes of Bacillus subtilis (61 %) and* Actinoplanes teichomyceticus *(62.4%). On the other hand, PKS-amplifying strains* Bacillus *BJ.7 and* Vibrio *MJ.5 showed closest sequence identity with polyketide synthase genes of* Bacillus subtilis *(73%)* and Anabaena sp 90 *(62%), respectively.*

Keywords: *Coral associated bacteria, Antimicrobial activity, PKS, NRPS*

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INTRODUCTION

Marine invertebrates that are mainly accumulating within coral reef ecosystems such as soft corals, sponges, tunicates, and bryozoans have long been recognised as the sources of structurally unique natural products. However, it has been suggested that natural products from marine invertebrates have stricking 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 bacteria in bacteria-invertebrate associations in the production of biologically active substances.

On the other hand, it has been well known that corals harbor diverse microbial communities (William *et al.* 1987; Shashar *et al.* 1994; Kim, 1994; Santavy *et al*. 1995; Kushmaro *et al.* 1996; Rohwer *et al.* 2001). Every surface immersed in the marine environment, including those organisms

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(Armstrong *et al.* 2001) such as muco-polysaccharides-covered coral surfaces providing a nutrient rich habitat for heterotrophic bacteria that leading to the formation of biofilm-forming microbial communities (Kushmaro *et al.* 1997).

Inhibitory interactions among coral-associated bacteria that occur on the coral surface then could be of great interest to search for secondary metabolite-producing bacteria. In addition, examination concernin secondary metabolite-producing bacteria among coral colonizers have been strongly neglected in comparison to soft-bodied marine invertebrates.

Polyketides and non-ribosomal peptides are two of the largest groups of multifunctional proteins that create a multitude of secondary metabolites (Hutchinson, 2003), many of them are used as therapeutic agents *et al.* 2001; Piel *et al.* 2003). Products of the microbial non-ribosomal peptide synthesis include the immunosuppressant cyclosporine and other antibiotics such as gramicin S, tyrocin A and surfactins (Kleinkauf and von Doehren, 1996). Among clinically important polyketides are the antibiotibiotic daunorubicin, erythromycin, lovastatin and rapamycin (Due *et al.* 2001).

With advanced techniques of molecular biology such as polymerase chain reaction (PCR), it is now become possible to carry out a screening on the presence of polyketides and non ribosomal peptides by using specific primers of polyketide synthases (PKS) (Piel, 2002) and non ribosomal polypeptide synthetases (NRPS) (Marahiel *et al.* 1997).

The present study focusses on marine bacteria, which are associated with the corals representing different life forms massive (*Porites lutea),* sub-massive (*Galaxea fascicularis),* branching *(Acropora* sp.) and folious *(Pavona* sp.) and produce secondary metabolites with antimicrobial activity. The examinations are coupled with PCR-based analysis for the presence of polyketide synthases and non-ribosomal polypeptide synthetases of the bacterial isolates.

MATERIALS AND METHODS

Sampling and isolation of coral-associated bacteria

Four different corals were collected from the vicinity of Panjang island, Jepara, North Java Sea, Indonesia by scuba diving and identified as *Porites lutea, Galaxea fascicularis, Acropora* sp. and *Pavona* sp. Upon collection coral fragments were put into sterile plastic bags (Whirl-Pak, Nasco, USA) and immediately brought to the Marine Station of the Diponegoro University where it was rinsed with sterile seawater and scraped off with a sterile knife. The resultant tissues were serially diluted in sterile seawater, spread on Marine Broth, (Difco, Becton Dickenson GmbH, Heidelberg, Germany) and incubated at 20°C for 48 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al.* 2000).

Inhibitory interaction tests

Inhibitory interaction tests of coral-associated bacteria against test bacteria were performed by using an overlay method. The following bacteria were used: *Escherichia coli* (DSM 498), *Staphylococcus lentus* (DSM 6672), *Bacillus subtilis* (DSM 347), and *Candida glabrata* (DSM 6425) obtained from the German Culture Collection (DSMZ, Braunschweig, Germany).

Overnight cultures of each target microorganism in the logarithmic phase (ca. 10^9 cells ml^{-1}) were mixed with TSB soft agar medium (TSB 3g, NaCl 10g and Bacto-agar 8g per liter). This mixture (1% v/v, except *C. glabrata* of 10% v/v) was poured on to the respective TSB medium surface, which was previously inoculated with coral-associated bacteria and incubated for 4 d at 20°C. The plates were then incubated at 20°C for 48 hours. Antimicrobial activity was defined by the formation of inhibition zones around the bacterial colonies.

PCR-based analysis of NRPS and PKS producing bacterial strains

To obtain genomic DNA of secondary metabolite producing-strains for PCR analysis, cell materials were 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´) (Marahiel *et al.*, 1997) and PKS degenerated primers KSDPQQF (5´-MGN GAR GCN NWN SMN ATG GAY CCN CAR CAN MG-3´) and KSHGTGR (5´-GGR TCN CCN ARN SWN GTN CCN GTN CCR TG -3´) domain (Piel, 2002). All primers were manufactured by MWG-Biotech (Ebersberg, Germany).
PCR was performed with an ProgeneThermal cycler (Techne, Burkhardtsorf, Germany) as follows: 1 µl template DNA, 1 µl of each of the appropriate primers, and 23 µl DNA free water (Fluka, Sigma-Aldrich Chemie GmbH, Germany) were added to puReTaq Ready-To-Go PCR beads (Amersham Biosciences Europe GmbH, Germany). The NRPS-PCR run comprised 40 cycles with denaturing conditions for 1 min at 95°C, annealing for 1 min at 70°C and extension for 2 min at 72°C, respectively. *Pseudomonas* sp. DSM 50117 was used as positive control. The amplification of PKS gene fragments included an initial denaturating step at 94°C for 2 min, followed by 45 cycles at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min. *Bacillus subtilis* 168 as utilized for positive control.

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 almost complete 16S rDNA sequences of strains were compared for homology to the NCBI GenBank and EMBL databases using BLAST (Basic Local Alignment Search Tool) and FASTA searches (Altschul *et al.*, 1990, Pearson 1990). Sequences were aligned using the program ClustalX Version 1.83 (Thomson *et al*. 1997). For phylogenetic calculations the PhyML software (Guindon and Gascuel, 2003) as well as the online version of PhyML (Guindon *et al*., 2005) were used. Trees were calculated by Maximum Likelihood (ML) method (Felsenstein, 1981) using the GTR model and estimated proportion of invariable sites as well as the Gamma distribution parameter.

Cloning and sequencing of (putative) peptide synthetase and polyketide domains

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).

DNA sequence accession numbers

The 16S rRNA gene sequences have been entered into the GenBank database under the sequence accession number AY000000-, the putative peptide synthetase and polyketide synthase sequences obtained under AM287210-AM287213.

RESULTS AND DISCUSSION

Results

Inhibitory interaction test

To estimate antimicrobial activity of coral associated-bacteria, inhibitory interaction tests between test strains and coral associated isolates were carried out. As shown in Table 1, a total of 13 bacterial isolates belonging to the genera *Arthrobacter* (1 strain), *Bacillus* (7 strains), *Micrococcus* (1 strain), *Pseudoalteromonas* (1 strain), *Pseudovibrio* (1 strain), and *Vibrio* (2 strain), were successfully for their inhibitorial effect against at least 1 test strain. All 13 coral derived strains inhibited the growth of the Gram-positive bacteria *B. subtilis*. All isolates but one *Pseudovibrio* isolate inhibited the second Gram-positive test strain, *S. lentus.* Two *Bacillus* strains showed an antibacterial activity against the Gram-negative test strain *E. coli*, and one *Bacillus* isolate showed an antifungal activity against the yeast *C. glabrata*.

Detection of NRPS and PKS gene fragments by PCR

PCR-based analysis using specific primers of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) revealed that the coral-associated bacterial strains *Bacillus* sp BM1.5 and *Micrococcus* sp BJB showed a PCR amplificate for NRPS. In addition, two other bacterial strains (*Bacillus subtilis* BJ.7 and *Vibrio coralliitycus* MJ.5) exhibited a PCR product of PKS..

Putative peptide synthetase and polyketide sequences.

Two PKS-amplifying bacterial strains (BJ.7 and MJ.5) had homologies with polyketide synthase genes from *Bacillus subtilis* and *Anabaena* sp 90 **(Table 2),** meanwhile the other 2 NRPS-amplifying strains (BM1.5 and BJB) showed similarity with peptide synthetase genes of *Bacillus subtilis* and *Actinoplanes teichomyceticus*, respectively **(Table 3).**

Discussion

In a study aimed at the search for antimicrobial compounds from the sea, we investigated bacteria associated with corals from North Java Sea, Indonesia. Our attention was focused on the examination of coral associated-bacteria by using growth inhibition tests followed by PCR-based approach for the occurrence of gene fragments of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) which are believed to be responsible for the biosynthesis of two main family of secondary metabolites, polyketides and peptides.

Microbes including bacteria play crucial ecological roles in many marine ecosystems such as tropical coral reefs. Unfortunately, studies on bacterial associations with corals have been much less extensive (Knowlton and Rohwer, 2003). Considering the intense interaction among coral colonizers, in particular for the competition for space and nutrient (Slattery *et al.* 2001), coral associated-bacteria have been considered as potential sources of biologically active compounds (Moore, 1999).

In our study, we show that the corals *Porites lutea, Galaxea fascicularis, Acropora* sp. and *Pavona* sp. offered promising potency as the source of coral associates with biological activities against *E. coli, S. lentus, B. subtilis* and *C. glabrata*. A total of 13 bacterial isolates belonging to Gram-positive genera, like *Bacillus*, *Micrococcus*, and *Arthrobacter*, and to Gram - negative genera, like *Pseudoalteromonas*, *Pseudovibrio* and *Vibrio* were found to inhibit the growth of at least 1 test strain.. It is interesting to note that 7 out of 13 active strains belonged to the genus *Vibrio* that were isolated from corals *Acropora* sp (2), *G. fascicularis* (3) and *Pavona* sp(2) but not from *P. lutea.* Furthermore, *Acropora* sp was found to dominate the existence of active strains including *Bacillus* (2), *Micrococcus* (1), *Arthrobacter* (1) and *Pseudovibrio* (1).

One isolate obtained from the coral *Pavona* sp. showed closest similarity to *Pseudoalteromonas* sp. The genus of *Pseudoalteromonas* is widely distributed in the marine environment (Alexeeva *et al.* 2003) and considered one of the most abundant group of marine γ-proteobacteria. For the survival in such environments, the members of genus *Pseudoalteromonas* have been known to develop metabolic pathways that produce biologically active metabolites. Extensive studies regarding the capability of *Pseudoalteromonas* to produce diverse secondary metabolites were reported (Austin, 1989; Jensen and Fenical, 1994; Holmstrom and Kjelleberg, 1999). Further work by (Reid *et al.* 1993; Deng *et al.* 1995) showed that *Pseudoalteromonas luteoviolacea*, owns a non-ribosomal peptide synthetase, which produces the siderophore alterobactin. Radjasa *et al.* (2007) reported the antibacterial activity of a bacterium *Pseudoaltermonas luteovioalacea* TAB4.2 isolated from coral *Acropora* sp. collected from the neighboring sampling site in the North Java Sea. A novel antimicrobial protein was also reported from a marine bacterium strain X153 which was closely related to *P. piscicida* (Longeon *et al.* 2004). Furthermore, Sobolevskaya *et al.* (2005) successfully isolated the recently decribed *Pseudoalteromonas maricaloris* KMM 635^T collected in the Coral Sea at a depth of 10 m that produces a brominated cyclic depsipeptides.

Hutchinson (2003) mentioned that microorganisms, especially ones that inhabit marine environments are noted for the ability to produce a wide range of chemicals known as secondary metabolites devoted to the property of self-defense, intercellular communication and other aspects of microbial life. Du *et al.* (2001) mentioned that polyketides and non-ribosomal peptides represent the largest group of natural products many of which are clinically important drugs. For instance, non-ribosomal peptides including cyclosporin, penicillin and vancomycin are synthesized by non-ribosomal peptide sythetases (NRPS) (Kleinkauf and von Doehren, 1990). On the contrary, polyketides such as daunorubicin, erythromycin, lovastin and rapamycin are derived from sequential condensation of short carboxylic acids.

In the present study, however, only 2 strains amplified NRPS and 2 other strains amplified PKS gene fragments B*acillus* sp BM1.5 showed closest similarity to the genes for peptide synthetase and protein building protein of *Bacillus subtilis.* Toh *et al*. (2004) reported the occurrence of emetic toxin of a foodborne pathogen, *Bacillus cereus* that is putatively a product of non-ribosomal peptide synthesis. The presence of NRPS gene fragment in coral bacterium *Bacillus* BM1.5 supported the fact that other *Bacillus* specieroduce peptides using non-ribosomal peptide synthesis (Stachelhaus and Marahiel, 1995). Meanwhile another bacterium associated with coral *Acropora* sp, *Bacillus subtilis* BJ.7 was found to amplify the gene fragment of PKS and showed sequence similarity of 73% to previously reported polyketide gene of *Bacillus subtilis* (acc. no. U11039). Scotti *et al*. (1993) studied the potential ability of *B. subtilis* to synthesize polyketides.

Micrococcus sp BJB, had sequence similarity to peptide synthetas of *Actinoplanes teichomyceticus*. The members of *Micrococcus* are heterotrophic bacteria distributed in various environments such as seawater (Tanaka *et al.* 2001), marine sediments (Zhong *et al.* 2002) and prawn-rearing water (Phatarpekar *et al.* 2002). Members of the genus *Micrococcus* have also the potency as producers of antimicrobial compounds. A marine bacterium *Micrococcus* MCCB 104 isolated from hatchery water demonstrated extracellular antagonistic properties against *Vibrio alginolyticus, V. parahaemolyticus, V. fluviallis, V. nereis, V. proteolyticus, V.*

mediterranei, *V. cholerae* and *Aeromonas* sp (Jayaprakash *et al*. 2005). Variacin, a bacteriocin produced by *Micrococcus varians* inhibited other Gram-posititve bacteria, but not Gram-negative ones (Pridmore *et al*, 1996).

Two antibacterial active isolates of vibrios, *V. coralliitycus* MJ.5 and *V. parahaemolyticus* MJ.11 were obtained from coral *Porites lutea*. Isolate *V. coralliitycus* MJ.5 also amplified the gene fragment of PKS and had sequence similarity (62%) to polyketide synthase of *Anabaena* sp90. Polyketide natural products are common metabolites of blue-green algae (cyanobacteria) (Burdja *et al*. 2001). Neilan *et al*. (1999) mentioned that *Cyanobacteria* produced a myriad array of secondary metabolites, including alkaloids, polyketides, and non ribosomal peptides, some of which are potent toxins. Isolate *V. parahaemolyticus* MJ.11 inhibited the growth of *Bacillus subtilis* and *Staphylococcus lentus*. *V. parahaemolyticus* has been reported as the member of *Vibrio*, a dominant genus responsible for the observed mortality in prawn-farmings (Lightner, 1988). As far as known it is uncertain, if *V. parahaemolyticus* is also a pathogen of corals. Cervino *et al*. (2004) considered that further studies should include the examination to determine if strains of *V. parahaemolyticus* and *V. alginolyticus* are capable of inducing disease signs in *Montastraea* spp.

CONCLUSION

Our study on coral-associated bacteria provides evidence of antimicrobial properties among coral colonizers. Although in minor numbers, the coral-associated bacteria might be capable of producing polyketides and peptides, because of the detection of NRPS and PKS gene fragments as well as their antimicrobial activities. The discovery of the potential for the synthesis of polyketides and peptides among coral-associated bacteria should be of interest because of promising applications in the development of pharmacological polyketides and peptides. Furthermore, considering the fact that research on coral-bacteria association has been fairly limited, the future study on the search for secondary metabolite producers among coral colonizers should be given prominence.

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