ANTIBACTERIAL PROPERTY OF A CORAL-ASSOCIATED BACTERIUM *PSEUDOALTEROMONAS LUTEOVIOLACEA* AGAINST SHRIMP PATHOGENIC *VIBRIO HARVEYI* (IN VITRO STUDY)

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ABSTRACT

A coral-associated bacterium was successfully screened for secondary metabolites production based on PCR amplification of the non-ribosomal peptide synthetase gene and was identified as closely related to *Pseudoalteromonas luteoviolacea* based on its 16S rDNA. The bacterium was found to inhibit the growth of shrimp pathogenic bacterium tested, *Vibrio harveyi*. To characterize the inhibiting metabolite, a 279 bp long DNA fragment was obtained and the deduced amino acid sequence showed conserved signature regions for peptide synthetases and revealed a high similarity to NosD (40% identity), a multifunctional peptide synthetase from *Nostoc* sp. GSV224, and NdaB (44% identity), a peptide synthetase module of *Nodularia spumigena*.

Keywords: coral-associated bacterium, molecular characterization, antibacterial activity, *Vibrio harveyi*

Introduction

The growth of shrimp farming spectacularly increased over the last 20 years because the governments and international development agencies promoted it, lured by enormous profits to be made. Furthermore, it has been estimated that half of the world's seafood demand could be met by aquaculture industries.

On the other hand, shrimp aquaculture production in much of the world is decreased by disease, particularly caused by luminous *Vibrio harveyi*. The high density of shrimps in tanks and ponds is conducive for the spread of pathogens, and the aquatic environment, with regular applications of protein-rich feed, is ideal for culturing the bacteria (Moriarty 1999).

It is a widely observed phenomenon that microbial cells attach firmly to almost any surface submerged in marine environments, grow, reproduce, and produce extracellular polymers that provide structure to the assemblage termed as biofilm (Kioerboe *et al.* 2003).

It is well understood that corals harbor diverse microbial communities (William *et al.* 1987; Shashar *et al.* 1994; Kim 1994; Kushmaro *et al.* 1996; Rohwer *et al.* 2001). Their surface is covered by muco-polysaccharides, which provides a matrix for bacterial

colonization leading to the formation of biofilm-forming microbial communities (Kushmaro *et al*. 1997).

Recently many coral-associated bacteria have been characterized as sources of marine natural products (Moore 1999), especially since the coral surface is more nutrient rich than seawater or even sediments (Unson *et al*. 1994; Bultel-Ponce *et al.* 1999). However, colonization of coral surfaces by bacteria and other microorganisms is mostly nondestructive to corals (Paul *et al*. 1986; Coffroth 1990; Kim 1994).

Due to the close spatial vicinity of these biofilm-forming bacteria, it can be expected that the indigenous microbial population is adapted to competitive conditions, e.g. for available nutrients and space (Slattery *et al*. 2001). The production of secondary metabolites is a common adaptation of these bacteria to compete in such microenvironments.

More information on coral-associated bacteria might be desirable, as many of these bacteria serve as sources of secondary metabolites including novel antibiotics. Here, we report on antibacterial property of a secondary metabolite-producing coral bacterium closely related to *Pseudoalteromonas luteoviolacea* against shrimp pathogenic bacterium *Vibrio harveyi*.

Materials and methods

Sampling and Isolation of Coral-associated Bacteria. The coral was collected from Teluk Awur (06°37'02,5'' N; 110°38'21,4" E), North Java Sea, Indonesia by scuba diving and identified as *Acropora* sp. according to Veron (1988). 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, 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).

PCR-based Screening of NRPS Producing Bacterial Strain. For PCR analysis, genomic DNA of strain TAB 4.2 was taken from cell material on an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-70 °C) and thaw (95 °C). Amplification of peptide synthetase gene fragments was carried out with the 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´) (MWG-Biotech, Ebersberg, Germany) designed from conserved regions of various bacterial peptide synthetase sequences from GenBank (Marahiel *et al.* 1997).

PCR was performed with an Eppendorf Mastercycler (Eppendorf Inc., Germany) as follows: 2 µl template DNA, 40 pmol of each of the appropriate primers, 125 µmol of each deoxyribonucleoside triphosphate, 5 µl of 10 x RedTaq™ PCR buffer (Sigma, Germany), 1.2 mg ml^{-1} (final concentration) bovine serum albumin (Sigma) and 0.75 unit RedTaq™ DNA polymerase (Sigma) were adjusted to a final volume of 50 µl with sterile water (Sigma). A 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.

Cloning and Sequencing of a (putative) Peptide Synthetase Domain. The amplified PCR-product was gel-purified using the Perfectprep™ Gel cleanup Kit (Eppendorf, Germany) and ligated into the pGEM-T vector (Promega, Germany) following the manufacturers protocol. Recombinant clones containing an insert were prepared using the DYEnamic Direct cycle sequencing kit (Amersham Life Science, Inc, UK) for subsequent sequencing on an automated DNA sequencer Model 4200 (LI-COR, Inc, UK). Both strands were sequenced twice using M13F and M13R labeled with IRDye™800 as sequencing primers (Messing 1983). Prior to further analysis of the gene fragment the primer sequences on both sides of the fragment were

removed. The deduced amino acid sequence of the gene fragment was compared for homology with BLAST search (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.* 1997).

PCR amplification and sequencing of 16S rRNA gene fragments. PCR amplification of the almost complete 16S rRNA gene of strain TAB4.2, purification of PCR products and subsequent sequencing analysis were performed according to the method of Brinkhoff and Muyzer (1997). The determined 1204 bp DNA sequence of strain TAB 4.2 was then compared for homology to the BLAST database.

Phylogenetic analysis. All sequences used were at least 1200 bp long. 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 ARB software package (http://www.mikro.biologie.tu-muenchen.de (Strunk *et al.* 1998).

DNA Sequence Accession Numbers. The 16S rRNA gene sequence of strain TAB4.2 has been entered into the GenBank database under the sequence accession number AY338404, the putative peptide synthetase sequence obtained from strain TAB4.2 under AY338405.

Inhibitory Interaction Test. Inhibitory interaction test of isolate TAB4.2 against pathogenic *Vibrio harveyi* obtained from the collection of Microbiogenetics laboratory, Department of Biology, Diponegoro University, was performed by using the agar disk-diffusion method (Conception *et al.* 1994). The *V. harveyi* strain was previously isolated from infected shrimp from shrimp pond of Center for Research on Brackish Water Aquaculture, Ministry of Marine Affairs and Fisheries, Jepara, Indonesia. 100 µl culture of *V. harveyi* in the logarithmic phase (ca. 10^9 cells ml^{-1}) was spread on to agar medium. Paper disks (Φ 8 mm; Advantec, Toyo Roshi, Ltd, Japan) containing 10 µl of the primer-carrying bacterial strain was placed on the respective agar surface. The plates were then incubated at room temperature for 48 hours. Antibacterial activity was defined by the formation of inhibition zones greater than 9 mm around the paper disk.

Results

PCR-based Screening and Inhibitory Interaction Test

PCR-based screening revealed that the coral-associated bacterial strain TAB4.2 was capable of producing secondary metabolites, in particular a non-ribosomal polypeptides. As indicated in Figure 1, bacterial strain TAB4.2 possesses the NRPS gene as represented by the occurrence of a single DNA band similar to the positive control on the agarose gel. Further inhibitory interaction test showed that strain TAB4.2 inhibited the growth of shrimp pathogenic *V. harveyi.*

DNA Sequencing and Phylogenetic Analysis

A comparison of the 16S rRNA gene sequence of strain TAB4.2 with sequences from GenBank demonstrated that this strain is affiliated to the family *Pseudoalteromonas* within the order *Alteromonadales*. The phylogenetic tree shown in Figure 2 indicating that isolate TAB 4.2 is most closely related with *Pseudoalteromonas luteoviolacea (*accession number X82144) with a homology of 98%.

Cloning and Sequencing of a (putative) Peptide Synthetase Domain

To investigate the genetic potential of strain TAB 4.2 to produce secondary metabolites, a 279 bp long DNA fragment was obtained. The deduced amino acid sequence indeed showed conserved signature regions for peptide synthetases. A comparison with proteins in the GenBank database revealed a high similarity to NosD (accession number AAF17281; 40 % identity), a multifunctional peptide synthetase from *Nostoc* sp. GSV224, and also to NdaB (accession number AAO64402; 44 % identity), a peptide synthetase module of *Nodularia spumigena.*

Discussion

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 coral-associated bacterium (TAB4.2) carrying the NRPS gene. This bacterium is 98% identical to *Pseudoalteromonas luteoviolacea* based on its 16S rRNA gene sequence. Alteromonadales and Vibrionales of the δ Proteobacteria were among the dominant producers of antibiotics on marine snow from the Southern California Bight (Long & Azam 2001).

Growth inhibition of *V. harveyi* by NRPS strain TAB4.2 was 11.75 ± 0.014 mm which demonstrates the so far uncharacterized secondary metabolites of strain TAB4.2 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 pathogenic bacterium *V. harveyi* by strain TAB4.2 may further reflect the potential role of coral bacteria in controlling shrimp disease. A further work is needed,however, to confirm the effectiveness this strain in the shrimp culture.

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). Products of the microbial non-ribosomal peptide synthesis include the immunosuppressant *cyclosporine* and other antibiotics such as *gramicin S, tyrocin A* and *surfactins* (Kleinkauf & von Dohren 1996).

The comparison of the derived amino acid sequence of the putative non ribosomal peptide synthetase of strain TAB4.2 revealed a high homology to sequence fragments of known peptide synthetases. Highest similarity was found with sequences of organisms belonging to the phylum *Cyanobacteria*, from which most genera possess non-ribosomal peptide synthetase genes (Christiansen *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.

Interestingly, the organism closest related to TAB4.2, *Pseudoalteromonas luteoviolacea,* owns a non-ribosomal peptide synthetase, which produces the *siderophore alterobactin* (Reid *et al.* 1993; Deng *et al.* 1995). Although the biological function of the gene product remains unknown, the feasibility that the respective gene detected in strain TAB 4.2 codes for a non ribosomal peptide synthetase is high.

In conclusion, a symbiotic coral bacterium *Pseudoalteromonas luteoviolacea* TAB4.2 carrying the a 279 bp long NRPS gene fragment accounts for its antibacterial activity against shrimp pathogenic bacterium *Vibrio harveyi*.

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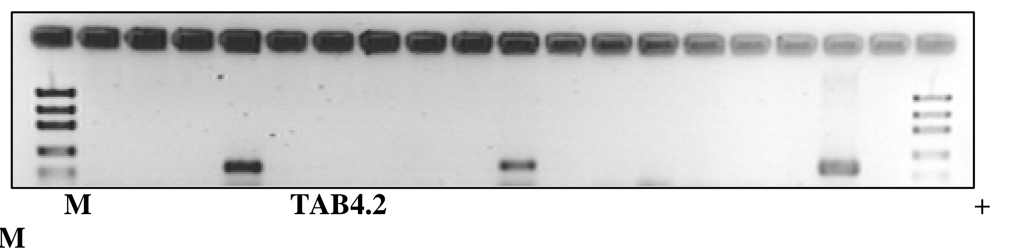
Figures and legends



M **TAB4.2** + **-**
M

Fig 1. PCR-based screening of NRPS producing-TAB4.2 strain.
+ control (*Pseudomonas fluorescens* DSM No. 50117); M: DNA markers.

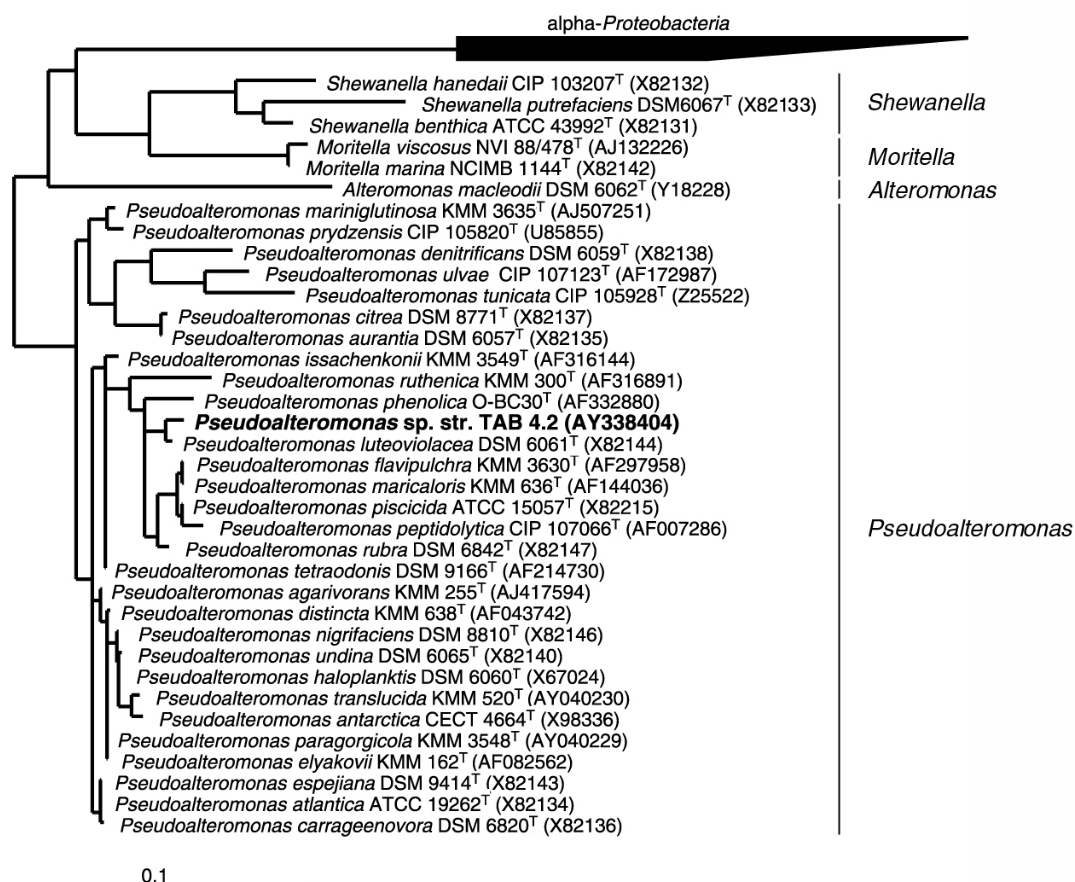


Fig 2. Phylogenetic tree based on comparative 16S rRNA gene sequence analysis of *Pseudoalteromonas* species showing the phylogenetic affiliation of strain TAB4.2. Selected sequences from the alpha subclass of *Proteobacteria* were used to root the tree. Accession numbers of the 16S rRNA gene sequences are given in parenthesis. The bar indicates 10% sequence divergence.