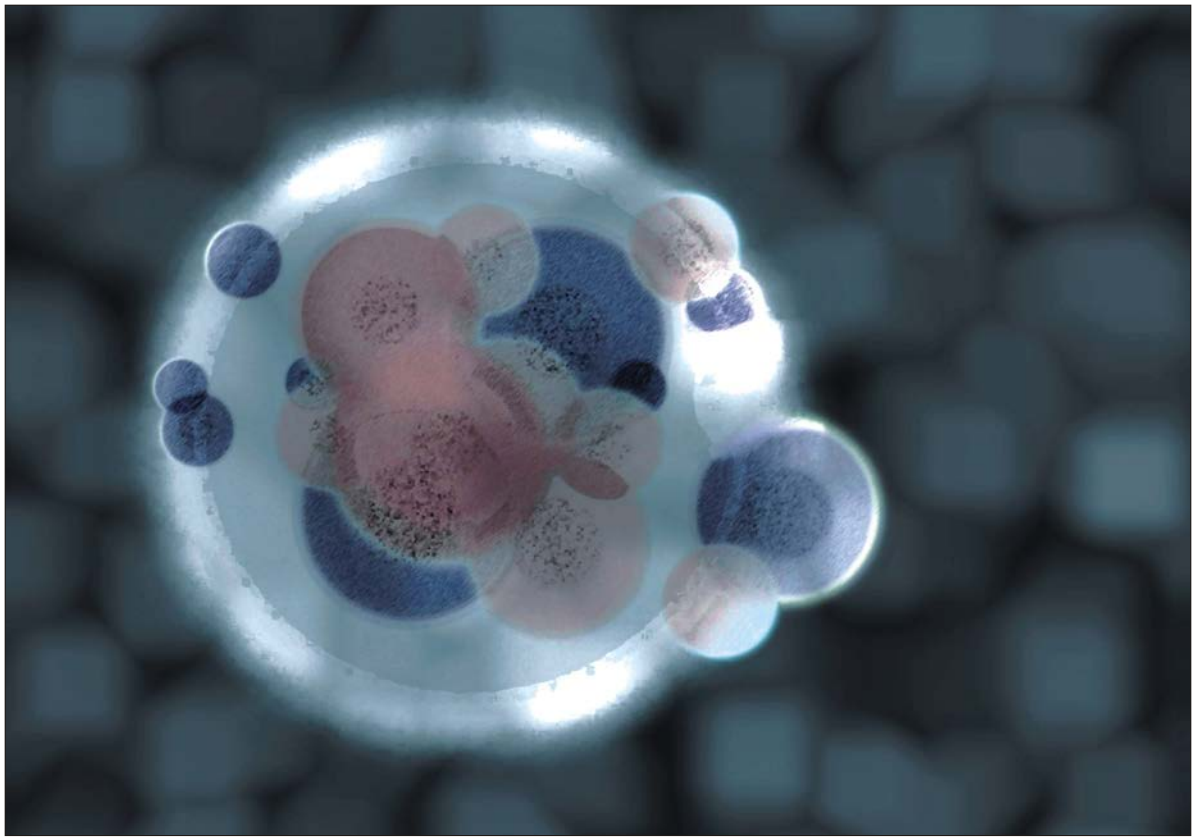




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Antagonistic Activity of a Marine Bacterium *Pseudoalteromonas luteoviolacea* TAB4.2 Associated with Coral *Acropora* sp.

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Abstract: A coral-associated bacterium was successfully screened for secondary metabolite production based on biological activity and PCR amplification of the non-ribosomal peptide synthetase (NRPS) gene and was identified as closely related to *Pseudoalteromonas luteoviolacea* based on its 16S rDNA. The bacterium was found to inhibit the growth of other coral-associated bacteria and pathogenic bacteria. 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*. To estimate the possible role of secondary metabolites, analyses on the quantitative proportion of *Pseudoalteromonas* was carried out. The results revealed that *Pseudoltermonas* group was indeed present within surfaces of coral *Acropora* sp.

Key words: Coral-associated bacterium, antagonistic activity, *Pseudoalteromonas*, *Acropora* sp.

INTRODUCTION

Coral reefs are the most diverse marine ecosystems, however, little is known about the microbial diversity in these ecosystems. 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 mucopolysaccharides, which provides a matrix for bacterial colonization leading to the formation of biofilm-forming microbial communities (Kushmaro *et al.*, 1997).

Marine organisms including those from coral reef ecosystems have become sources of great interest to natural product chemistry, since they provide a large proportion of bioactive metabolites with different biological activities (Faulkner, 2000). In particular, marine invertebrates with high species diversity in the Indo-Pacific regions (Coll and Sammarco, 1986) are often rich in secondary metabolites and are preferential targets in the search for bioactive natural products (Sammarco and Coll, 1992).

Perhaps the most significant problem that has hampered the investigation of secondary metabolites is their low concentration (Munro *et al.*, 1999). In marine

invertebrates many highly active compounds contribute to <10⁻⁶% of the body-wet weight (Proksch *et al.*, 2002). Providing sufficient amounts of these biologically active substances, hence, may be a difficult task. Limited amounts found in the producing organism, limited quantities of the organism itself and geographic or seasonal variations in the produced secondary metabolites (Kelecom, 2002), further complicate the study of secondary metabolites of aquatic organisms.

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 (Kiorboe *et al.*, 2003). 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 (Long and Azam, 2001; Grossart *et al.*, 2004). Many coral-associated bacteria have been characterized as sources of a great variety 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). Hence, colonization of coral surfaces by bacteria and other microorganisms is beneficial for specific microbial assemblages and is mostly nondestructive to the coral host (Paul *et al.*, 1986; Coffroth, 1990; Kim, 1994).

Recently, PCR amplification of degenerate primers targeted to sequences of genes essential in the biosynthesis of particular secondary metabolites has been used to estimate the genetic ability of microorganisms to produce various compounds belonging to Non-ribosomal peptide synthetases (NRPS) (Marahiel *et al.*, 1997; Ayuso-Sacido and Genilloud, 2004), polyketide synthases (PKS) (Metsa-Ketela *et al.*, 2002; Piel *et al.*, 2003) and halogenases (Pirae and Viing, 2002).

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.

Due to cultivation biases only a minor fraction of heterotrophic microorganisms in the coral reefs has yet been isolated. 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 isolation, screening and characterization of a novel secondary metabolite-producing coral bacterium closely related to *Pseudoalteromonas luteoviolacea*.

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 (Fig. 1) by scuba diving and identified as *Acropora* sp. according to Veron (1986). 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 h. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al.*, 2000).

Screening of coral bacteria with biological activity: To screen their biological activity, coral bacterial isolates were tested against two indicator microorganisms (*Escherichia coli* and *Staphylococcus aureus*). One 100 µL culture of each indicator microorganism in the logarithmic phase (ca. 10^9 cells mL⁻¹) were spread on to agar medium. Several paper disks (8 mm; Advantec, Toyo Roshi, Ltd., Japan) containing 10 µL of the coral bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 h. Biological activity was defined by the formation of inhibition zones around the paper disk. Isolate showed biological activity against indicator microorganisms

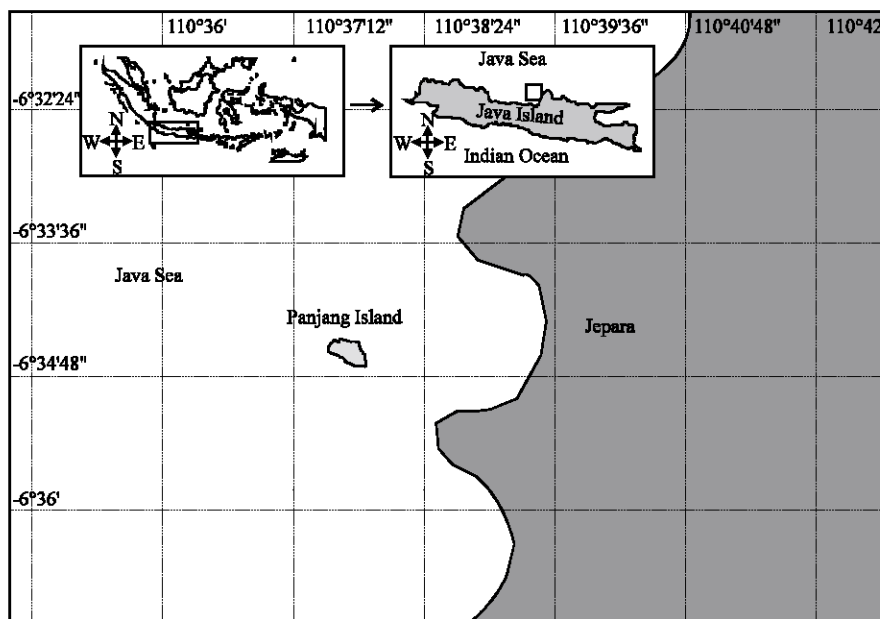


Fig. 1: Sampling site for the collection of coral from Teluk Awur water, Jepara

was chosen for further screening based on PCR technique by using specific primers of Non-ribosomal peptide synthetase (NRPS).

Inhibitory interaction tests: Inhibitory interaction tests of isolate TAB4.2 against other bacteria were performed by using the agar disk-diffusion method. The following bacteria associated with *Acropora* sp. were used: *Salinicoccus roseus*, *Oceanobacillus iheyensis*, *Halomonas salina*, *Bacillus iodinum* and *Silicibacter lacuscaeruensensis*. Pathogenic bacteria used were *Staphylococcus aureus*, *Escherichia coli*, *Vibrio harveyi*, *V. parahaemolyticus* and *V. anguillarum*. One 100 μ L culture of each target microorganism in the logarithmic phase (ca. 10^9 cells mL^{-1}) were spread on to agar medium. Several paper disks (8 mm; Advantec, Toyo Roshi, Ltd., Japan) containing 10 μ L of the primer-carrying bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 h. Antibacterial activity was defined according to modified method of Burgess *et al.* (2003) by the formation of inhibition zones greater than 9 mm around the paper disk.

PCR -based screening of NRPS producing bacterial strain and natural DNA: To obtain genomic DNA of strain TAB 4.2 for PCR analysis, cell material was taken from 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 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 (Marahiel *et al.*, 1997).

DNAs from in situ microbial community were conducted by using Soil DNA extraction kit. Extracted DNAs were used to determine the diversity of unculturable coral bacteria. PCR was performed with an Eppendorf Mastercycler (Eppendorf Inc., Germany) as follows: 2 μ L template DNA, 40 pmol of each of the appropriate primers, 125 μ mol of each deoxyribonucleoside triphosphate, 5 μ L of 10 x RedTaqTM PCR buffer (Sigma, Germany), 1.2 mg mL^{-1} (final concentration) bovine serum albumin (Sigma) and 0.75 unit RedTaqTM DNA polymerase (Sigma) were adjusted to a final volume of 50 μ L with sterile water (Sigma). A PCR run comprised 40 cycles with denaturing conditions for 1 min at 95°C , annealing for 1 min at 70°C and extension for two minutes at 72°C , respectively.

Cloning and sequencing of a (putative) peptide synthetase domain:

The amplified PCR-products of bacterial strain was gel-purified using the PerfectprepTM 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 Dynamic 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 IRDyeTM800 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: Phylogenetic analysis was performed with the ARB software package (<http://www.mikro.biologie.tu-muenchen.de>) (Strunk *et al.*, 1998). 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.

The 16S rRNA gene sequence of strain TAB4.2 has been entered into the GenBank database under the sequence accession number AY33 8404, the putative peptide synthetase sequence obtained from strain TAB4.2 under AY33 8405.

RESULTS

Screening of coral bacteria with biological activity:

Screening among coral bacteria associated with coral *Acropora* sp. by using indicator microorganisms revealed that only one isolate, TAB4.2 that was capable of inhibiting the growth of both *Escherichia coli* and *Staphylococcus aureus*, while the rest of isolates showed activity against indicator microorganisms.

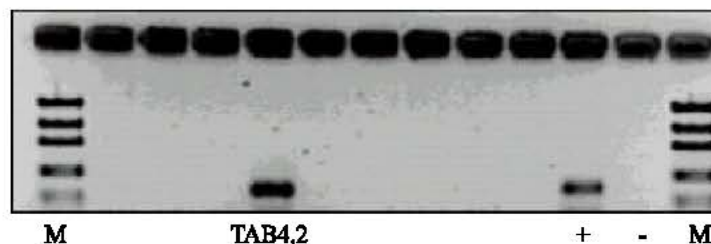


Fig 2: PCR-based screening of NRPS producing-TAB4.2 strain. The control (*Pseudomonas fluorescens* DSM No. 50117) is shown by a plus sign; No addition of DNA extract is shown by a negative sign; tracks marked M are DNA markers

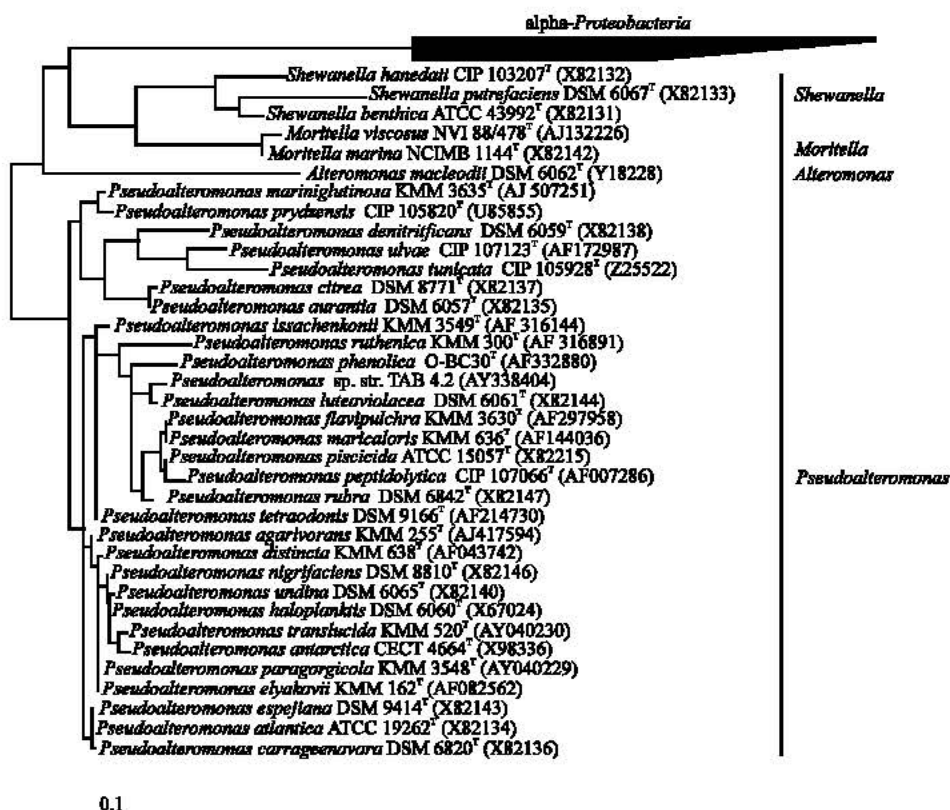


Fig 3: Phylogenetic tree based on comparative 16S rRNA gene sequence analysis of *Pseudoalteromonas* species showing the phylogenetic affiliation of strain TAB4.2. Selected sequences from the alpha subclass of *Proteobacteria* were used to root the tree. Accession numbers of the 16S rRNA gene sequences are given in parenthesis. The bar indicates 10% sequence divergence

Table 1: Inhibitory interaction of bacterial strain TAB4.2 against coral bacteria and pathogenic bacteria

Coral bacteria	Antibacterial activity	Pathogenic bacteria	Antibacterial activity
<i>Bacillus iodinum</i>	+	<i>Escherichia coli</i>	+
<i>Salinococcus roseus</i>	+	<i>Staphylococcus aureus</i>	+
<i>Silicibacter latuscaerueus</i>	+	<i>Vibrio parahaemolyticus</i>	+
<i>Oceanobacillus iheyensis</i>	+	<i>Vibrio harveyi</i>	+
<i>Halomonas salina</i>	+	<i>Vibrio anguillarum</i>	+

Table 2: Diversity of clones associated with coral *Acropora* sp.

Clone	Length	Closest relative	Homology	Accession No.
ACP-1	324	<i>Uncultured Enterobacteriaceae</i>	98	AY635978
ACP-2	444	<i>Vibrio alginolyticus</i> NRL-SS41	98	AY357910
ACP-3	463	<i>Enterobacter asburiae</i>	97	AY310732
ACP-4	481	<i>V. alginolyticus</i> NRL-SS41	98	AY357910
ACP-5	484	<i>V. parahaemolyticus</i> clone	98	AF388387
ACP-6	444	<i>Pseudoalteromonas</i> sp. 01/121	99	AJ874345
ACP-7	518	<i>V. alginolyticus</i> NRL-SS41	93	AY357910
ACP-8	356	<i>V. alginolyticus</i> NRL-SS41	98	AY357910
ACP-9	503	<i>Pseudoalteromonas</i> sp. Md 236	97	AY461673
ACP-10	488	<i>V. parahaemolyticus</i> Vp16	97	AF388387
ACP-11	522	<i>V. parahaemolyticus</i>	98	BA000032
ACP-12	595	<i>V. parahaemolyticus</i>	99	BA000032
ACP-13	529	<i>V. alginolyticus</i> NRL-SS41	98	AY357910
ACP-14	423	<i>V. parahaemolyticus</i> Vp16	97	AF388387
ACP-15	501	<i>Enterobacter</i> sp. TUT 1014	98	AB098582
ACP-16	439	<i>V. alginolyticus</i> NRL-SS41	98	AY357910
ACP-17	468	<i>Uncultured Enterobacteriaceae</i>	97	AY635978
ACP-18	323	<i>Uncultured Enterobacteriaceae</i>	96	AY635978
ACP-19	502	<i>Vibrio natriegus</i>	95	AJ874352
ACP-20	373	<i>Uncultured clone</i> SBRBC2	97	AY444158
ACP-21	572	<i>Pseudoalteromonas</i> sp. 01/121	98	AJ874345
ACP-22	488	<i>Vibrio parahaemolyticus</i> MP-2	99	AY9 11391
ACP-23	467	<i>Pseudoalteromonas</i> sp. 01/121	98	AJ874345
ACP-24	524	<i>V. alginolyticus</i> NRL-SS41	98	AY357910
ACP-25	313	<i>Pseudoalteromonas</i> sp. 01/121	98	AJ8 74345
ACP-26	408	<i>V. parahaemolyticus</i> clone Vp16	95	AF388387
ACP-27	446	<i>V. alginolyticus</i> NRL-SS41	98	AY357910
ACP-28	436	<i>Arthrobacter nicotianae</i>	98	AJ3 15492
ACP-29	495	<i>Uncultured Enterobacteriaceae</i>	99	AY635978
ACP-30	313	<i>Pseudoalteromonas</i> sp. 01/121	98	AJ874345

Inhibitory interaction tests: To estimate antimicrobial activity of strain TAB4.2 and its biotechnological potential, inhibitory interaction tests with other coral-associated and pathogenic bacteria were carried out. Table 1 shows that strain TAB4.2 inhibited the growth of all tested bacteria.

PCR-based screening: PCR-based screening further revealed and supported that the coral-associated bacterial strain TAB4.2 was capable of amplifying gene fragments of non-ribosomal peptide synthetase (NRPS). As indicated in Fig. 2, 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.

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 Fig. 3 shows that isolate TAB 4.2 is most closely related with *Pseudoalteromonas luteoviolacea* (accession number X82 144) with a homology of 98%.

Putative peptide synthetase sequence: To investigate the genetic potential of strain TAB4.2 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*.

Diversity of uncultured bacteria: A total of 30 clones were selected and sequenced. The estimated diversity of the yet uncultured community associated with coral

Acropora sp. is presented in Table 2. It can be seen that the member of genera *Vibrio*, *Pseudoalteromonas*, *Arthrobacter* and *Enterobacter* were present among coral colonizers. In particular, *Vibrio* had the highest relative abundance among clones (56%), whereas *Pseudoalteromonas* and the member of Enterobacteriaceae shared the same number of 6 clones, respectively. Minor members were closely related to *Arthrobacter nicotianae* and clone *SBRBC2*.

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 Proteobacteria were among the dominant producers of antibiotics on marine snow from the Southern California Bight (Long and Azam, 2001) and the German Waddensea (Grossart *et al.*, 2004).

Growth inhibition of coral-associated bacteria by NRPS strain TAB4.2 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. This assumption is supported by the fact that our NRPS positive strain, TAB4.2 exhibited antibacterial activity against all tested bacteria. The efficient inhibition of pathogenic bacteria by strain TAB4.2 may be beneficial not only for the respective bacterium but also for the coral host since it further protect the coral from infection (Rohwer *et al.*, 2002). Estimated diversity of unculturable microorganisms associated with coral *Acropora* sp. further supported the existence of the member of *Pseudoalteromonas* among coral colonizers. Although this group was not the dominant part, at least this raise the possibility the role of secondary metabolites produced by this group in enhancing their evolutionary success in coral surfaces. 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 and von Doehren, 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.

The occurrence of structurally related peptides in diverse microorganisms might be due to horizontal gene transfer events of biosynthetic clusters (Kleinkauf and von Doehren, 1996). 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 TAB4.2 codes for a non ribosomal peptide synthetase is high. The present study highlights the production of secondary metabolites by a presumably symbiotic coral bacterium (TAB4.2) 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). Although further work is needed to clarify the isolated bioactive substances produced by TAB4.2, hence, the application of molecular approach through PCR using specific NRPS primers provides detection of microorganisms with high pharmaceutical potential and is suitable to greatly improve the screening efficiency for secondary metabolite-producer among coral-associated bacteria.

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