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Molecular Phylogeny and Modular Structure of Hybrid NRPS/PKS Gene Fragment of *Pseudoalteromonas* sp. NJ6-3-2 Isolated From Marine Sponge *Hymeniacidon perleve*

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Among 12 marine bacterial strains from the China coast that exhibited interesting bioactivity (positive for both antimicrobial and cytotoxic activities), only four strains, namely, NJ6-3-1, NJ6-3-2, NB-6, and YTHM-17, had a KS domain or A domain when screened for PKS and NRPS genes using a PCR. Interestingly, two of these strains belonging to *Pseudoalteromonas* and associated with the marine sponge *Hymeniacidon perleve* were positive for both PKS and NRPS, whereas the other two strains of *Pseudoalteromonas* did not have a PKS or NRPS gene. A molecular phylogeny analysis and DGGE analysis of the *Pseudoalteromonas* sp. indicated that they had a specific affinity with the host marine sponge *Hymeniacidon perleve*. Furthermore, an analysis of a partial sequence of *Pseudoalteromonas* sp. NJ6-3-2 isolated from the marine sponge *Hymeniacidon perleve* obtained from genomic walking using a computational approach indicated a relatively complete PKS module including auxiliary domains (DH, KR, and Cy).

Keywords: Marine bacteria, polyketide synthase, nonribosomal peptide synthetase, *Pseudoalteromonas* sp.

Studies of natural marine products, such as sponges, have yielded numerous drug candidates with intriguing pharmacological mechanisms targeting ion channels, enzyme inhibition, microtubule stabilization, and DNA intercalation [6]. In recent years, the investigation of marine natural products has moved to the microscopic level, due to numerous findings of similar structures between marine microorganisms and macroorganisms, such as marine sponges and ascidians, indicating that microorganisms would seem to be a better biological source to tackle the supply problem of marine invertebrates [17]. Moreover, the structural characteristics of marine natural products have revealed that they mainly belong to two important chemical families, namely, polyketides and cyclopeptides, and are synthesized by multifunctional enzymes called polyketide synthases (PKSs) and nonribosomal peptide synthases (NRPSs) [7]. With the rapid development of molecular genetics related to polyketides and nonribosomal peptides in microorganisms, the biosynthetic origins of marine natural products, such as onnamide from the marine sponge *Theonella swinhoei,* bryostatin from bryozoan *Bugula neritina*, and patellamide from marine ascidian *Lissoclinum patella*, have been unequivocally confirmed in symbiotic microorganisms in several studies based on the genomic approach [3, 16, 19].

Marine invertebrates, especially marine sponges, have been found to harbor large numbers of diverse bacteria in their tissue based on 16S rRNA sequencing studies [25, 21]. However, the real challenge is how to identify the true microbial source for the biosynthesis of the target natural product from the host. For example, discodermolide, originally isolated from the marine sponge *Discodermia dissolute*, is considered to be biosynthesized from bacterial type I modular polyketide synthase [20]. However, metagenomic analyses have indicated a huge diversity of polyketide synthase and nonribosomal synthase gene clusters in microorganisms associated with marine sponges [18].

In the course of screening marine bioactive bacteria from coastal areas off China, 12 strains with both antimicrobial and cytotoxic activities were selected from hundreds of isolated colonies [26, 12], and four of these strains noted to belong to the genus *Pseudoalteromonas*, which is ecologically significant for a marine environment. The genus *Pseudoalteromonas* is generally found in association with marine eukaryotes and contains numerous marine species that synthesize biologically active molecules [8]. Thus, studies of such associations can elucidate the mechanisms important in microbe–host interactions. More recently, many *Pseudoalteromonas* species have been demonstrated to produce antibacterial products,

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which appear to aid them in the colonization of surfaces, including those of their hosts, along with the production of agarase, toxin, and bacteriolytic substances, which assist the bacterial cells in competing for nutrients and space, while protecting against surface-grazing predators [9]. For example, Isnansetyo and Kamei [10] found that *Pseudoalteromonas phenolica* produces a phenolic compound with anti-MRSA bioactivity, whereas Franks *et al*. [4] identified a bipyrrole pigment from *Pseudoalteromonas tunicate* with antimicrobial activities.

However, this raises two major scientific questions: (1) the existence of a host specificity for microorganisms that produce particular secondary metabolites with possible ecological or pharmacological importance and (2) the effective selection of a specific bioactive microorganism strain, if culturable, based on the existence of a secondary metabolite biosynthetic gene. One feasible approach may be to combine the bioactivity screening and secondary metabolite biosynthetic gene screening. Accordingly, this study attempted to provide molecular evidence of the host specificity of *Pseudoalteromonas* isolated from a marine sponge using the PKS phylogeny and a DGGE analysis. Furthermore, molecular characterization of the PKS domain in *Pseudoalteromonas* NJ6-3-2 isolated from the marine sponge *Hymeniacidon perleve* was carried out based on a 10 kb region in a PKS probe flanking sequence.

Materials and Methods

Collection and Processing of Marine Samples

The 12 strains with bioactivity were previously isolated by the current authors [26]. Four different higher marine organisms, including *Hymeniacidon perleve*, *Homoiodoris japonica*, *Berenicea ampulliformis*, and *Sargassum thunbergii*, were collected from the intertidal zone during low tide at coastal locations near Nanji Island (East China Sea, Zhejiang Province, China). One tropical marine sponge, *Phakellia fusca*, was collected from the intertidal zone during low tide at a coastal location near Sanya (South China Sea, Hainan Province, China). Four dinoflagellates, *Alexandrium catenella, Karlodinium micrum, Scrippsiella trochoidea,* and *Prorocentrum micans*, came from the laboratory collection of the current authors.

DNA Extraction from Environmental Samples and Pure Cultures

The nucleic acids in the environmental samples were extracted using a FastDNA spin kit for soil (Qbiogene, U.S.A.) according to the manufacturer's instructions. The method involved mechanical cell lysis by bead beating (FastPrep DNA extractor; Qbiogene, U.S.A.), followed by selective DNA adsorption to microporous silicate filters. The DNA was washed with ethanol in the presence of chaotropic salts and finally eluted in a low-salt buffer. The genomic DNA from the pure bacteria that showed significant bioactivity was then extracted using a Bacterial Genomic DNA Extraction Kit (TIANGEN, China) according to the manufacturer's instructions.

Design of Oligonucleotide Primers

To identify the cytotoxic bacteria strains, the 16S rDNA gene from the strains was amplified using the universal primers 27F and 1492R (Table 1) [24]. Amplification of the A regions, approximately 1,000 bp in size, was performed using the degenerate oligonucleotide primers NPRSF and NRPSR (Table 1) [15]. The detection of modular PKS genes was performed using degenerate oligonucleotide primers. The primers were designed based on conserved regions, where the downstream primer was based on the conserved region HGTG, whereas the upstream primer was based on the conserved region AMDPQQR after comparison with known ketosynthase sequences in GenBank using the multiple alignment program CLUSTAL W. The specificity of the degenerate oligonucleotides KSF and KSR (Table 1) was tested against all DNA sequences available in GenBank using the program FASTA. KS domains, approximately 680 bp in size, were detected by the degenerate PCR in the marine bacteria with cytotoxicity. For the *Pseudoalteromonas*-specific DGGE analysis, the *Pseudoalteromonas*-specific primers Psalt815R with a GC-clamp and Eub341F (Table 1) were used to amplify fragments sized about 500 bp [23].

Table 1. Primers used in this study.

Primer	Target	Sequence(5'-3')
27F	16S rDNA	AGAGTTTGATCCTGGC
1492R	16S rDNA	GGTTACCTTGTTACGACTT
Eub341F[a]	*Pseudoalteromonas* sp	CCTACGGGAGGCAGCAG
Psalt815R	*Pseudoalteromonas* sp	CCAGCTTCTAGTAGACATCGTT
NPRSF	A domain	GCNGGYGGYGCNTAYGTNCC
NRPSR	A domain	CCNCGDATYTTNACYTG
KSF	KS domain	GCGATGGATCCNCAGCAGCG
KSR	KS domain	GTGCCGGTNCCGTGNGYYTC

[a]The DGGE primer had a GC clamp attached to its 5' end: 5'-CGCCCGC-CGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCCCGCCCG-3'

PCR Amplification

The DNA preparations were used as the template DNA for *Taq* polymerase. All the PCRs were performed in a T1 Thermal Cycler (Biometra, Germany), and the products screened using 1% agarose gel electrophoresis. The following reaction mixtures and conditions were used to amplify the 16S rDNA gene, NRPS A and PKS KS domains, and *Pseudoalteromonas* gene with the use of the primers shown in Table 1. (i) For the 16S rDNA gene PCR, the reaction mixture consisted of 35.9 μl of ddH_2O, 5 μl of 10×*Taq* buffer, 3 μl of 25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (TaKaRa, Japan), 1 μl of 10 mM deoxynucleoside triphosphates, 2 μl of 25 μM 1492R primer, 2 μl of 25 μM 27F primer, and 0.7 μl of the DNA template. The 16S rRNA gene PCR was then run with the following holds and cycles: 94°C for 5 min; 35 cycles at 94°C for 45 s, 54°C for 45 s , and 72°C for 1 min; plus 72°C for 7 min. (ii) For the NRPS and PKS PCRs, the reaction mixture consisted of 38.1 μl of ddH_2O, 5 μl of 10×*Taq* buffer, 3 μl of 25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (TaKaRa, Japan), 1 μl of 10 mM deoxynucleoside triphosphates, 1 μl of 50 μM NRPSF or KSF primer, 1 μl of 50 μM NRPSR or KSR primer, and 0.5 μl of the DNA template. The reactions were run with the following holds and cycles: 94°C for 3 min; 35 cycles at 94°C for 1 min, 50°C for 1 min for NRPS or 59°C for 1 min for PKS , and 72°C for 2 min for NRPS or 72°C for 1 min for PKS; plus 72°C for 7 min. (iii) For the *Pseudoalteromonas* PCR for the DGGE analysis, the reaction mixture consisted of 35.9 μl of ddH_2O, 5 μl of 10×*Taq* buffer, 3 μl of 25 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (TaKaRa, Japan), 1 μl of 10 mM deoxynucleoside triphosphates, 2 μl of 25 μM

Eub341F or Psalt815R primer, and 0.7 µl of the DNA template. The reactions were run with the following holds and cycles: 94°C for 5 min; 35 cycles at 94°C for 45 s, 62°C for 45 s, and 72°C for 1 min; plus 72°C for 7 min.

***Pseudoalteromonas*-specific DGGE Analysis**

The DGGE was carried out using a Dcode Universal Detection System instrument and gradient former 475 according to the manufacturer's instructions (Bio-Rad, U.S.A.). The DGGE system was used with an 8% acrylamide gel and denaturating gradient ranging from 25% to 65%. The 100% denaturant solution contained 7 mol/l urea, 40% (v/v) formamide, 8% acrylamide/bisacrylamide, and a 1×TAE buffer (pH 8.0) in ultrapure water. Meanwhile, the 0% denaturant solution contained 8% acryamide/bisacrylamide and a 1×TAE buffer (pH 8.0) in ultrapure water. The concentration of ammonium persulfate and TEMED was 0.03% (w/v) and 0.15% (v/v), respectively. The PCR samples were mixed with a loading dye (0.08%, w/v, bromophenol blue; 0.08%, w/v, xylene cyanol, and 30%, v/v, glycerol) and transferred to the bottom of the well. The DGGE was optimized to run at a constant voltage of 200 V for 7 h at 60°C for maximum band separation. The gels were attained using the silver dye method [2].

Genomic Walking

To confirm the existence of a PKS cluster in NJ6-3-2, genomic walking was used to find unknown genomic DNA sequences adjacent to the KS sequence obtained from NJ6-3-2 by PCR amplification. The genomic walking of NJ6-3-2 was performed using a BD GenomeWalker universal kit (Clontech, U.S.A.) according to the manufacturer's instructions.

Cloning and DNA Sequencing

The cloning of the PCR products was performed with a TA cloning kit according to the manufacturer's instructions (TaKaRa, Japan), following purification from 1% agarose gels using a QIAquick gel extraction kit (QIAGEN, Holland). Thereafter, the PCR product insertion was tested in 10 random clones as follows: each selected colony was placed in 100 µl ddH_2O, heated at 95°C for 10 min, and centrifuged at 3,000 rpm for 5 min, and then the supernatant was used as the crude plasmid template for a PCR. The inserts were amplified from the plasmid templates using standard M13 multiple-cloning site primers and PCR conditions. The *Escherichia coli* strains possessing plasmids with unique fragments of the predicted sizes were grown for 18 h in Super Broth at 37°C, and the plasmids extracted using a Plasmid Miniprep Kit I (Omega Bio-tek, U.S.A.). The sequencing was performed using the M13F and M13R reverse primers, Big Dye Terminators volume 3.1, and an ABI PRISM 373 analyzer (SANGON, China).

Sequence Analysis

For NRPS and PKS, the nucleotide sequences were translated into peptide sequences using BioEdit version 5.0.7. The percentage similarity and identity to other translated sequences were then determined using BLAST in conjunction with the National Center for Biotechnology Information (NIH, MD, U.S.A.). To construct a phylogenetic tree, the nucleotide sequences of interest were aligned with other sequences obtained from GenBank using Clustal X [22], and unrooted neighbor-joining phylogenies constructed using MEGA version 3.1 with a bootstrap consisting of 100 replications [11]. For the NJ6-3-2 sequences obtained from the genomic walking, the screening of open reading frames (ORF) was performed using the Glimmer v3.02 online software at the NCBI Web site. The ORF sequences were then translated into peptide sequences using BioEdit version 5.0.7. and analyzed using Web-based software NPRS-PKS (http://btisnet.nii.res.in/~zeeshan/searchnrps.html) [14] to screen the large multienzymatic, multidomain megasynthases involved in the biosynthesis of NPRS or PKS.

GenBank Accession Numbers

The sequences presented in this study have been deposited in GenBank under the accession numbers AY621063, AY626827, AY626829~AY626838, DQ666948~DQ666952, DQ873696, DQ873698, DQ873697, DQ873699, DQ986329, EU556341, EU556342, and EU556343.

RESULTS

Screening of NRPS and PKS Genes

Nonribosomal peptide synthase and polyketide synthase are biosynthetic systems involved in the synthesis of a large number of important biologically active compounds produced by microorganisms. Thus, to assess the presence of these biosynthetic systems in the isolated bioactive bacteria, PCR primers were designed to specifically amplify the A domains of the NRPS, and KS domains of PKS. The results showed that putative NRPS A domains were successfully amplified in 33% (4 of 12) of the DNA templates from the marine bacteria with bioactivity (Table 2). However, no NRPS A domains were amplified from NJ1-1-1, NJ5-1-2B, QD5-2-1, YTBM8-1A, ATCI02-4, NB-52, SSN-6, and SS6-4. Moreover, no putative PKS KS domains were amplified from any of the marine bacterial extracts, except for NJ6-3-1 and NJ6-3-2 (Table 2).The negative results from the initial NRPS and PKS PCRs were also confirmed through subsequent annealing-temperature gradient NRPS and PKS PCRs.

To verify that the amplified products were NRPS A and PKS KS domains, the amplified fragments were cloned and sequenced. Ten clones were then selected at random from the products of each cloning reaction for further analysis.

Table 2. Screening results for PKS and NRPS.

Bacteria	Most related genus	PKS	NRPS
NJ1-1-1	*Agrobacterium* sp.	–	–
NJ6-3-2	*Pseudoalteromonas* sp.	+	+
NJ5-1-2B	*Pseudoalteromonas* sp.	–	–
NJ6-3-1	*Pseudoalteromonas* sp.	+	+
QD5-2-1	*Paracoccus* sp.	–	–
YTBM8-1A	*Rheinheimera* sp.	–	–
YTHM-17	*Aerococcus* sp.	–	+
ATCI02-4	*Bacillus* sp.	–	–
NB-6	*Bacillus* sp.	–	+
NB-52	*Exiguobacterium* sp.	–	–
SS6-4	*Pseudoalteromonas* sp.	–	–
SSN 6	*Alteromonas* sp.	–	–

–, no PCR product; +, correct PCR product.

Table 3. Sequence alignment of KS and A domains of marine bacteria isolated in this experiment with those submitted to GenBank.

Marine bateria	Domain	Size/bp Accession No.	Most related species (Accession No.)	Sequence similarity/%
NJ6-3-1	KS	686 bp DQ666948	*Saccharophagus degradans* (ABD82980)	69
	A	1,004 bp DQ666950	*Myxococcus xanthus* (YP631805)	43
NJ6-3-2	KS	683 bp DQ666949	*Nitrosospira multiformis* (YP412520)	56
	A	1,007 bp DQ666951	*Pseudoalteromonas tunicata* (EAR30010)	75
NB-6	A	971 bp DQ873696	*Nostoc punctiforme* (ZP00110699)	45
YTHM-17	A	1,028 bp DQ666952	*Bacillus weihenstephanensis* (ZP01182547)	93

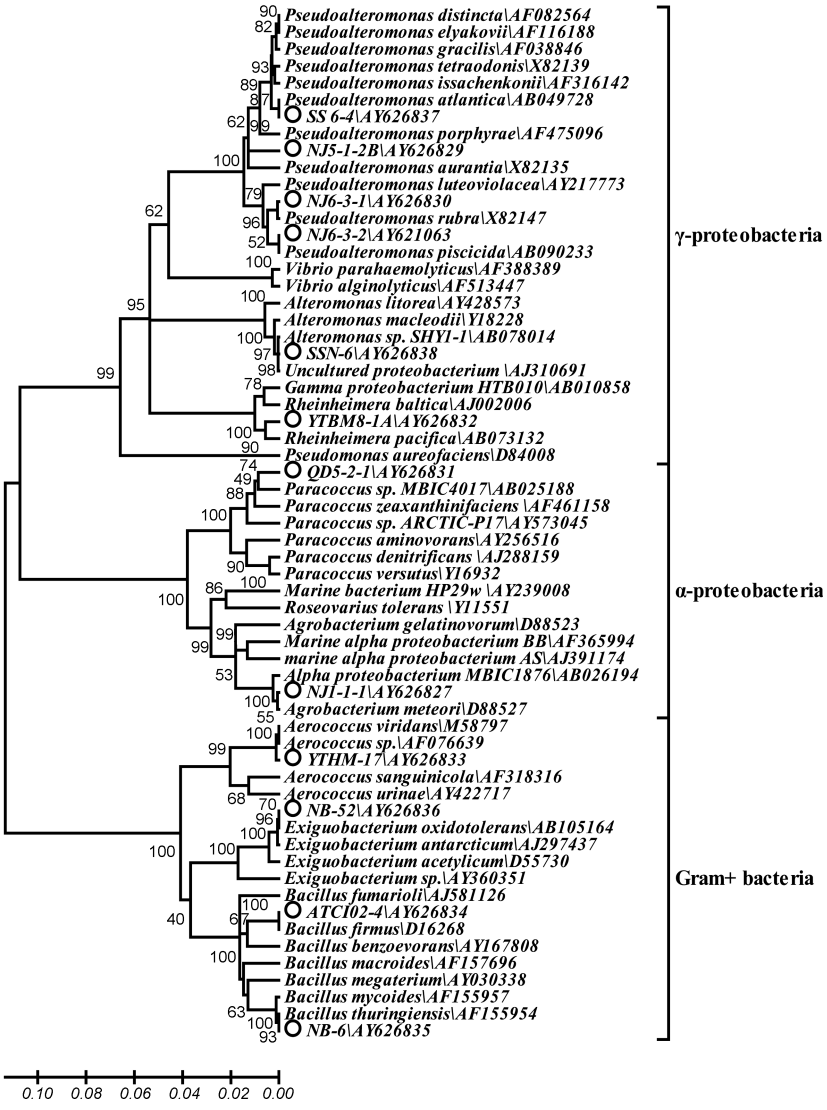

Fig. 1. Phylogenetic analysis of marine bacteria with bioactivities based on 16S rDNA sequences.
The phylogenetic trees were constructed using bootstrapping and the neighbor-joining rules. The numbers indicate the bootstrap confidence values as a percentage of 100 bootstrap replications, and values less than 40% are not shown. The sequences in this experiment are preceded by open circles.

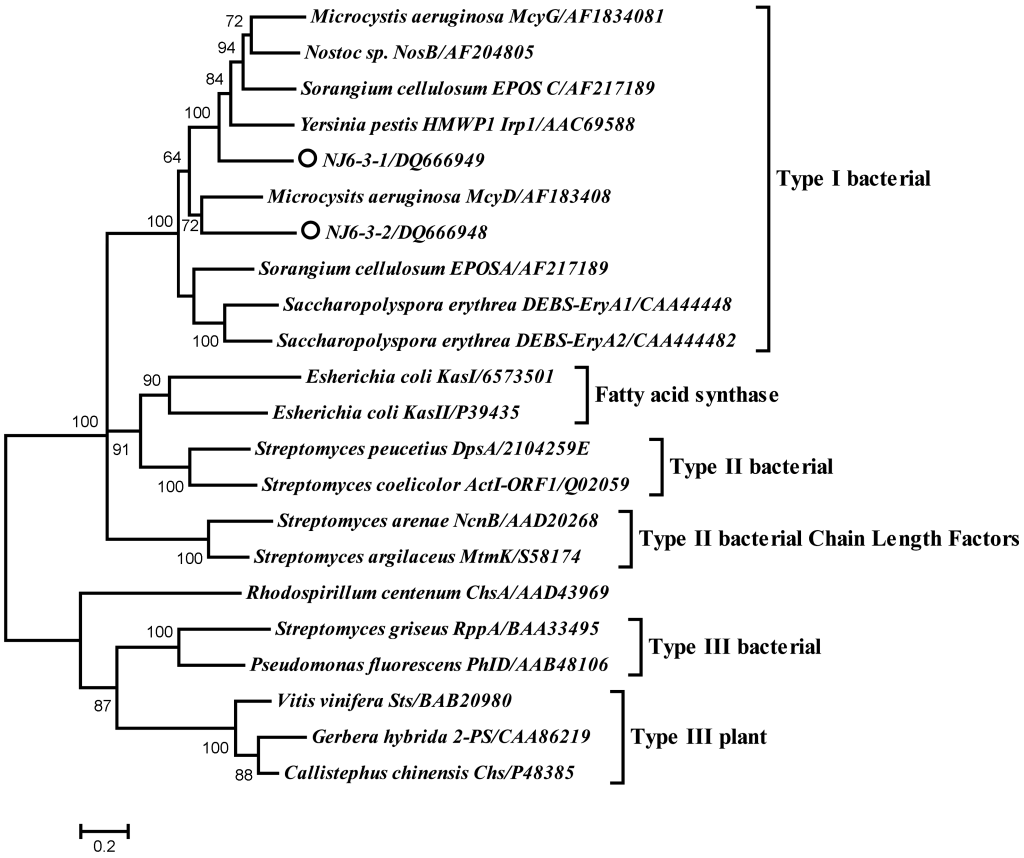

Fig. 2. Phylogenetic analysis of ketosynthase regions with respect to the diverse range of ketosynthase domains, including types I, II, and III.

The phylogenetic trees were constructed using bootstrapping and the neighbor-joining rules. The numbers indicate the bootstrap confidence values as a percentage of 100 bootstrap replications, and values less than 40% are not shown. The sequences in this experiment are preceded by open circles.

All the unique clones of the appropriate size (~1 kb for NRPS A domains; ~680 bp for PKS KS domains) were sequenced. A BLAST analysis in GenBank (tBlastn) showed that the putative PKS KS fragments from NJ6-3-1 and NJ6-3-2 exhibited the highest similarity (69% and 56%, respectively) to the KS fragments of *Saccharophagus degradan* and *Nitrosospira multiformis,* respectively (Table 3). Meanwhile, the putative NRPS A fragments from NJ6-3-1, NJ6-3-2, NB-6, and YTHM-17 exhibited sequence similarity to the A fragments of *Myxococcus xanthus, Pseudoalteromonas tunicata*, *Nostoc punctiforme,* and *Bacillus weihenstephanensis*, respectively. In particular, the sequences of YTHM-17 exhibited a high similarity (93%) to those of *Bacillus weihenstephanensis* already present in GenBank (Table 3).

Structural Analysis of KS and A Domains

PKSs have been divided into three main subclasses depending on the structure of the KS domains, namely, type I PKSs, type II PKSs, and type III PKSs [5]. Thus, the phylogeny of the KS domains in the marine bacteria with cytotoxity was investigated to determine their structural similarity to other well-described polyketide and fatty acid KS domain sequences from bacteria, fungi, and plants in GenBank (Fig. 2). As expected, three distinct clades were formed, representing type I, type II, and type III KS domains. The phylogenetic tree showed that the KS domains from NJ6-3-1 and NJ6-3-2 were both type I.

The KS domain is the most highly conserved domain in PKSs, and the conserved sequence motif VDTACSSSLVA was identified in all the KS domains analyzed. When analyzing the active-site sequence motif, it was found that the valine located 4th in the N-terminal, and leucine located 4th in the C-terminal of the cysteine active site, were both replaced by an isoleucine in the KS domain of NJ6-3-2, whereas the active-site sequence motif in the KS domain of NJ6-3-1 was found to belong to a distinct group of amino acid starter units in mixed or hybrid systems (Fig. 3). In this group, aspartate, the 3rd amino acid in the N-terminal of the cysteine active site is replaced by glutamine. This functionally distinct group of KS domains also contains a conserved sequence motif, NDKD, from the 22nd amino acid in the N-terminal of the cysteine active site. Although the role of these conserved motifs is unknown, they may be responsible for the KS enzymatic function or substrate recognition.

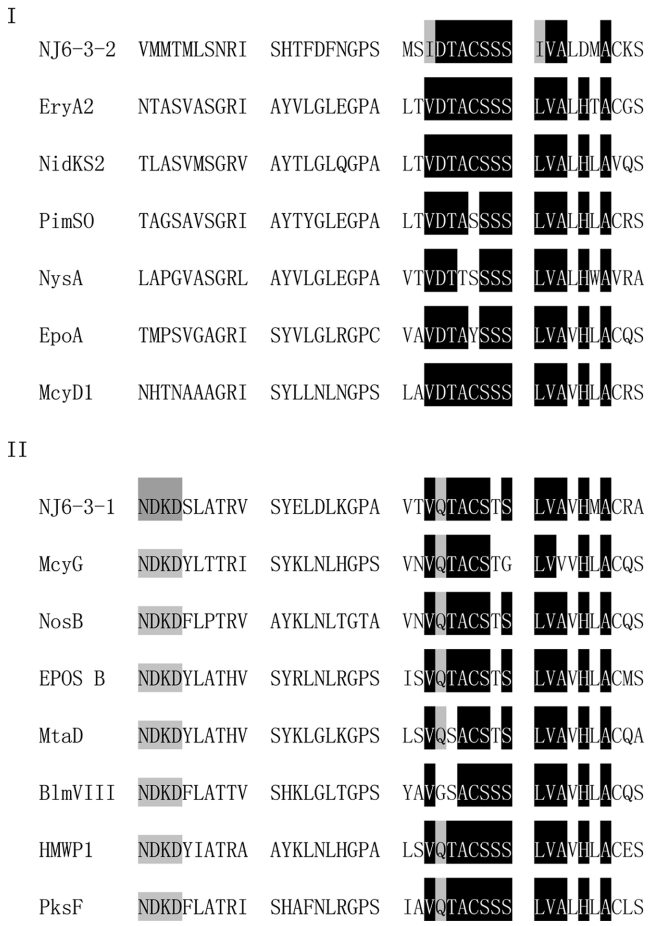

Fig. 3. Alignment of active sites of type I KS domains with distinct functions.

The conserved residues are highlighted in black. The residues conserved within each functional group are highlighted in gray. **I**. Representatives of general type I bacterial KS domains. The sequence is from NJ6-3-2 isolated from *Hymeniacidon perlev*e (DQ666948). Other aligned sequences are EryA2 from *Saccharopolyspora erythrea* (CAA44448), NidKS2 from *Streptomyces caelestis* (AF016585), PimSO from *Streptomyces natalensis* (AJ278573), NysA from *Streptomyces noursei* (AF263912), EpoA from *Sorangium cellulosum* (AF217189), and McyD1 from *Microcystis aeruginosa* (AF183408). **II**. Representatives of KS domains specific for amino acid starter units in mixed or hybrid systems. The sequence analyzed here is from marine bacterium NJ6-3-1 isolated from seawater (DQ666949). Also analyzed are McyG from *Microcystis aeruginosa* (AF183408), NosB from *Nostoc* sp. (AF204805), EPOS B from *Sorangium cellulosum* (AF217189), MtaD from *Stigmatella aurantiaca* (AAF19812), BlmVIII from *Streptomyces verticillus* (AAG02357), HMWP1 from *Yersinia pestis* (AF109251), and PksF from *Mycobacterium leprae* (S73015).

To analyze the potential function of the NRPS A domains from the marine bacteria, a phylogenetic tree was created in comparison with the A domains of antibiotics of nonribosomal peptides, including syringomycin, bacitracin, pyochelin, fengycin, and tyrocidin (Fig. 4). The unrooted NJ tree showed that the A domains from NJ6-3-1, NJ6-3-2, NB-6, and YTHM-17 were close to the A domains from syringomycin, where the A domains from NB-6, NJ6-3-1, and model 1 of syringomycin were in the same subclade. Meanwhile, the

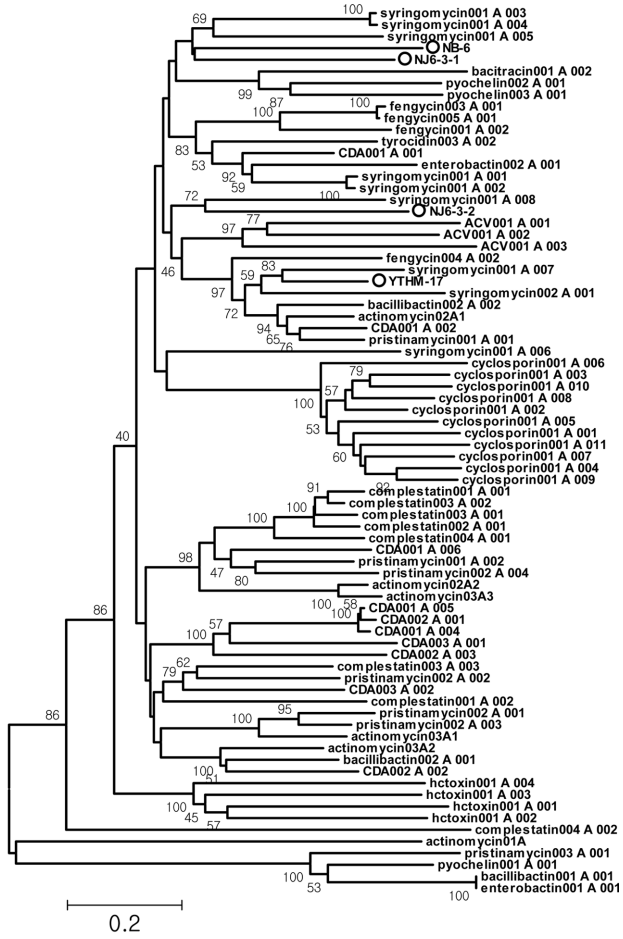

Fig. 4. Phylogenetic analysis of adenylation (A) domains based on a comparison with adenylation domains of partial antibiotics of nonribosomal peptides.

The phylogenetic trees were constructed using bootstrapping and neighbor-joining rules. The numbers indicate the bootstrap confidence values as a percentage of 100 bootstrap replications, and values less than 40% are not shown. The sequences in this experiment are preceded by open circles.

A domains from NJ6-3-2 and YTHM-17 were found to be similar to the 8th and 7th A domains in model 1 of syringomycin, respectively.

Molecular Phylogeny and DGGE Analysis Based on KS Domain of *Pseudoalteromonas* sp. NJ6-3-2

When screening for the PKS and NRPS genes, four *Pseudoalteromonas* strains produced very different results. Two of the strains isolated from the marine sponge *Hymeniacidon perleve*, namely, NJ6-3-1 and NJ6-3-2, were positive, whereas the other two isolated strains were negative (Table 2), indicating the possibility of a specific relationship between the two positive strains and the host. Thus, a phylogenetic tree was created to compare the KS domains from different organisms (Fig. 5). The unrooted NJ tree showed that the KS domain from NJ6-3-2 was very similar to the KS domain from the marine sponge *Hymeniacidon perleve*. Meanwhile, the KS

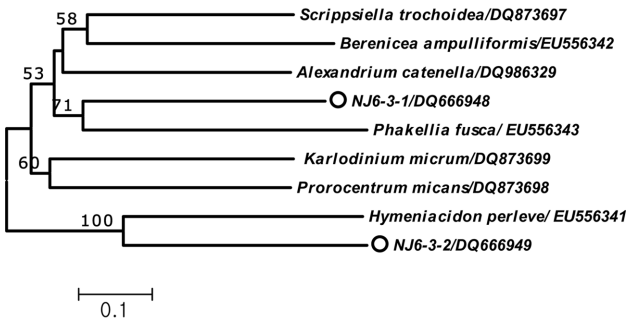

Fig. 5. Phylogenetic analysis of ketosynthase regions from different organisms.
The numbers indicate the bootstrap confidence values as a percentage of 100 bootstrap replications, and values less than 40% are not shown. The sequences in this experiment are preceded by open circles.

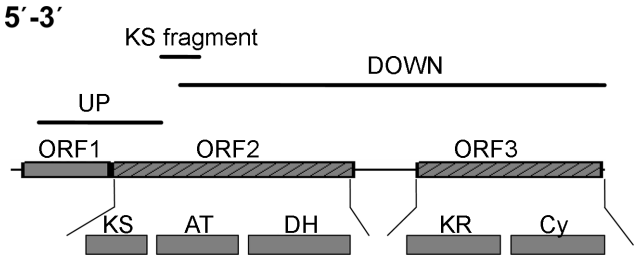

Fig. 7. Genetic organization of *Pseudoalteromonas*. sp. NJ6-3-2 PKS module from genomic walking.
The line through the map represents the KS fragment from *Pseudoalteromonas* sp. NJ6-3-2 in this study. The UP and DOWN lines represent the upstream and downstream sequences, respectively, of the KS fragment obtained by genomic walking. In the map, the cross-hatching indicates ORFs containing PKS domains. KS: ketosynthase domain; AT: acyltransferase domain; DH: dehydratase domain; KR: ketoreductase domain; Cy: cyclization domain.

domain from NJ6-3-1 was close to the KS domain from another marine sponge species *Phakellia fusca*. Furthermore, a DGGE analysis based on the KS domain was also carried out, where four different field samples (*Berenicea ampulliformis*, *Hymeniacidon perleve*, *Homoiodoris japonica*, and *Sargassum thunbergii*) collected from the same place as the originally isolated *Pseudoalteromonas* strains and one pure culture sample (*Pseudoalteromonas* sp. NJ6-3-2) were amplified using a *Pseudoalteromonas* genus-specific PCR (Fig. 6.). The DGGE analysis revealed bands for all the samples, except for the sample of *Sargassum thunbergii*. The migration positions of the bands for the pure cultures of *Pseudoalteromonas* sp. NJ6-3-2 were very similar to the samples of *Hymeniacidon perleve* and *Berenicea ampulliformis*, indicating a close relationship between *Pseudoalteromonas* sp. NJ6-3-2 and its marine sponge host.

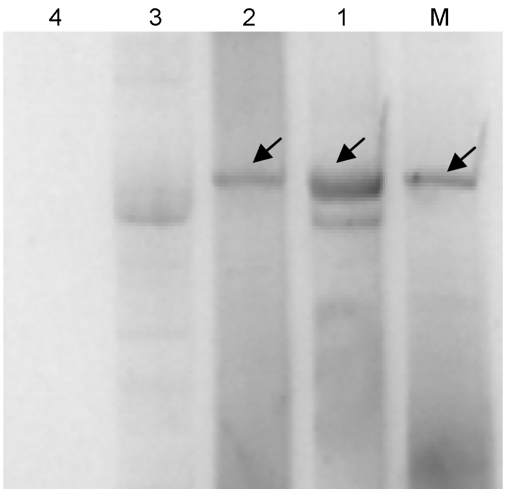

Fig. 6. DGGE patterns of *Pseudoalteromonas*-specific fragments.
M, marine bacterium NJ6-3-1; Lane 1, *Hymeniacidon perleve*; Lane 2, *Berenicea ampulliformis*; Lane 3, *Homoiodoris japonica*; Lane 4, *Sargassum thunbergii.*

Sequence Analysis of *Pseudoalteromonas*. sp. NJ6-3-2 Gene from Genomic Walking

To further confirm the existence of a PKS cluster in *Pseudoalteromonas*. sp. NJ6-3-2, genomic walking was used to amplify the flanking genomic DNA sequence to the KS sequence obtained from NJ6-3-2 by PCR amplification. A 10-kb continuous DNA sequence was obtained by five stepwise walking upstream and downstream of the KS fragment, respectively. Three open reading frames (ORFs) were also predicted using the Glimmer v3.02 online software at the NCBI Web site. Thus, to screen the large multidomain megasynthases involved in the biosynthesis of PKS, the sequences of the three ORFs were translated into peptide sequences using BioEdit version 5.0.7 and analyzed using the Web-based software NPRS-PKS (http://btisnet.nii.res.in/~zeeshan/searchnrps.html) [14]. The results of the sequence analysis are shown graphically in Fig. 7. With the exception of ORF1, the other two ORFs, ORF2 and ORF3, were found to encode ketosynthase (KS), acyltransferase (AT) and dehydratase (DH), and ketoreductase (KR) and cyclization (Cy), respectively, in domains related to the PKS module. As a result, a relatively complete PKS module, including auxiliary domains (DH, KR, Cy), was obtained. However, no PKS or NRPS module was previously found in the genome sequence of *Pseudoalteromonas atlantica*, which was uniquely sequenced in the genus of *Pseudoalteromonas* because of its production of acidic extracelluar polysaccharides during biofilm formation that show potential for element recycling, detoxification, and material production (http://genome.jgi-psf.org/finished_microbes/pseat/pseat.home.html).

DISCUSSION

To test the hypothesis that marine bioactive bacteria have a high probability of secondary metabolite biosynthetic genes, the PKS and NRPS genes from 12 marine bacterial strains that

had previously tested positive in antimicrobial and cytotoxic bioassays were screened using degenerative oligonucleotide primers [27]. A molecular phylogenetic tree based on the 16S rDNA sequences showed that the 12 strains contained a wide taxonomic diversity, belonging to α-proteobacteria, γ-proteobacteria, and Gram-positive bacteria, and included eight genera, such as *Agrobacterium, Pseudoalteromonas, Bacillus, Paracoccus, Rheinheimera, Aerococcus, Exiguobacterium,* and *Alteromonas*. However, the screening results indicated that only two strains were positive for both PKS and NRPS, whereas another two strains were positive for just NRPS. Therefore, in this case study, the results did not support the hypothesis that marine bioactive bacteria should contain PKS- or NRPS-specific genes. On the contrary, the diverse taxonomy of the 12 tested strains implied diverse structures for the secondary metabolites with possible other biosynthetic genes. Thus, polyketide and nonribosomal peptides were not the major structural classes for the tested strains. Hence, the combination of bioactivity screening with secondary metabolite biosynthetic gene screening to identify the biosynthetic gene in marine bioactive bacteria also needs to take account of conserved sequences from other biosynthetic pathways for PCR screening.

Four of the tested strains, namely, NJ6-3-1, NJ6-3-2, NJ5-1-2B, and SSN-6, were found to belong to the genus *Pseudoalteromonas* (Fig. 1), which is generally found in association with marine eukaryotes [8]. Many *Pseudoalteromonas* species have already been demonstrated to produce antibacterial products, which appear to aid them in the colonization of surfaces, including those of their hosts, along with the production of agarase, toxin, and bacteriolytic substances, which assist the bacterial cells in competing for nutrients and space, while protecting against surface-grazing predators [9]. Thus, it was very intriguing that two of the strains (NJ6-3-1 and NJ6-3-2) identified as belonging to the genus *Pseudoalteromonas* and associated with the marine sponge *Hymeniacidon perleve* were PKS and NRPS positive, whereas the other two strains (NJ5-1-2B and SSN6) did not include PKS or NRPS genes (Table 2). Therefore, the existence of a PKS or NRPS gene cluster in a strain may indicate a specific affinity with the host. Previous studies have shown that for survival and adaptation to the living environment, some bacteria and their host form a specific affinity with mutual benefits: the host supplies the associated bacteria with necessary nutrition, such as vitamins and nutrients, while the associated bacteria excrete certain products, such as antibiotics, to improve the chemical defensive capacity of the host [1]. Thus, revealing an affinity between a specific bacterial strain and its host organism is a prerequisite to elucidate the ecological importance of secondary metabolites in microbe–host interactions. A molecular phylogenic and DGGE analysis of *Pseudoalteromonas* sp. based on their KS domains showed that the KS domain from NJ6-3-2 was very similar to the KS domain from *Hymeniacidon perleve* (Figs. 5 and 6). Therefore, such results, although not conclusive, at least provide direct molecular evidence of a host specificity between *Pseudoalteromonas* NJ6-3-2 and the marine sponge *Hymeniacidon perleve*, from which the bacterial strain was isolated. Thus, a further study on the expression of antimicrobial activity by host-specific *Pseudoalteromonas* will be carried out.

For further molecular characterization of the putative PKS gene cluster in *Pseudoalteromonas* sp. NJ6-3-2, a 10-kb region of the PKS probe flanking sequence was obtained by gene walking. A computational approach for analyzing and predicting the domain organization of uncharacterized NRPS/PKS clusters, with the help of Web-based software, NRPS-PKS (http://www.nii.res.in/nrps-pks.html), was applied [14]. An analysis of the ORFs obtained from the gene walking sequence showed a relatively complete PKS module, including a distinct core and auxiliary catalytic domains. The core catalytic domains included a ketosynthase (KS) domain and acyltransferase domain (AT), whereas the auxiliary domains included a ketoreductase (KR) domain and dehydratase (DH) domain. Moreover, another auxiliary domain, cyclization (Cy), was also identified in the gene walking sequence, which is generally present in an NPRS module [13]. When considering that *Pseudoalteromonas* sp. NJ6-3-2 was found to include both a PKS KS domain and an NRPS A domain (Table 2 and 3), it is possible this strain may contain a mixed or hybrid PKS/NRPS multienzyme system that can synthesize secondary metabolites with a hybrid structure.

In conclusion, *Pseudoalteromonas* strain NJ6-3-2 isolated from the marine sponge *Hymeniacidon perleve* not only contained a NRPS/PKS gene, but also exhibited a specific affinity with its host. Moreover, a hybrid NRPS/PKS modular structure in *Pseudoalteromonas* sp. NJ6-3-2 was demonstrated by a computational analysis of the ORFs after genomic walking.

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