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ORIGINAL PAPER

Phylogenetic analysis of type I polyketide synthase and nonribosomal peptide synthetase genes in Antarctic sediment

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Abstract The modular polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) have been found to be involved in natural product synthesis in many microorganisms. Study on their diversities in natural environment may provide important ecological insights, in addition to opportunities for antibacterial drugs development. In this study, the PKS and NRPS gene diversities in two coast sediments near China Zhongshan Station were studied. The phylogenetic analysis of amino acid (AA) sequences indicated that the identified ketosynthase (KS) domains were clustered with those from diverse bacterial groups, including *Proteobacteria*, *Firmicutes*, *Planctomycetes*, *Cyanobacteria*, *Actinobacteria*, and some uncultured symbiotic bacteria. One new branch belonging to hybrid PKS/NRPS enzyme complexes and five independent clades were found on the phylogenetic tree. The obtained adenylation (A) domains were mainly clustered within the *Cyanobacteria* and *Proteobacteria* group. Most of the identified KS and A domains showed below 80 and 60% identities at the AA level to their closest matches in GenBank, respectively. The diversities of both KS and A domains in natural environmental sample were different from those in sewage-contaminated sample. These results revealed the great diversity and novelty of both PKS and NRPS genes in Antarctic sediment.

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Introduction

Microorganisms are the largest reservoir of potentially valuable natural compounds, such as polyketides, nonribosomal peptides, and alkaloids. Over the last 50 years, natural compounds produced by microorganisms, especially the *Actinobacteria*, have been extensively used to develop most of the antibacterial drugs proposed by pharmaceutical companies (Ginolhac et al. 2004). However, the detection of new compounds from natural environment has become more and more difficult in spite of the efforts to increase both screening capacities and the number of bacteria tested (Faulkner 2000, 2002; Watve et al. 2001), because previously described strains and molecules are being rediscovered and redescribed (Firn and Jones 2000). Two efforts have been undertaken to improve the situation in recent years. One is the introduction of meta-genomic techniques, which can obtain the gene clusters for natural products directly from those abundant uncultured microorganisms in the natural environment. The functional analysis of meta-genomic library has revealed many new compounds (Gillespie et al. 2002), new polyketide synthase (PKS) (Courtois et al. 2003; Moffitt and Neilan 2003), and even new functions like a membrane-associated proteolytic system (Beja et al. 2000). Another approach is to use cultured microbial isolates from extreme or unusual habitats (such as the deep sea, hot springs or habitats of extreme cold) since the microbial community composition is likely to contain unusual and phylogenetically divergent microorganisms with unique adaptations to their habitats, which might be correlated in some cases with synthesis of unusual

natural products, and would also tap into unexplored new microbial sources of natural products and the gene for their synthesis (Pathom-Aree et al. 2006). And besides, the microorganisms living in extreme environment are still largely unknown.

Most of the screening for the new bioactive natural compounds is focused on the detection of novel modular PKSs and nonribosomal peptide synthetases (NRPSs) which have been found to be involved in natural product synthesis in many bacteria, fungi, and plants (Cane et al. 1998; Moffitt and Neilan 2003). The PKSs and NRPSs synthesize a diverse array of biologically active compounds, including antibiotics, toxins, siderophores, and immunosuppressants (Cane et al. 1998; Crosa and Walsh 2002). Both are large (200–2,000 kDa) multi-functional enzymes that possess modular organization, implicated in the biosythesis of compound structures including pharmaceutically and industrially important bioactive compounds and being engineered for the combinatorial biosynthesis of natural products (Cane and Walsh 1999). A typical modular PKS is composed of acyltransferase, ketosynthase (KS), and acyl carrier protein domains (Schwarzer and Marahiel 2001; Staunton and Weissman 2001). The KS domain is responsible for the condensation of an extender unit onto the growing polyketide chain during polyketide biosynthesis, and is involved in the production of a structurally diverse range of metabolites (Moffitt and Neilan 2003). Analogous to the PKS gene module, an NRPS module usually contains an adenylation (A) domain, a peptidyl carrier protein domain, and a condensation domain (Schwarzer and Marahiel 2001). The A domain selects the cognate amino acid (AA) from the pool of available substrates and generates the corresponding aminoacyl adenylate using ATP (Stachelhaus et al. 1999).

It has been shown that the replacement of a domain or module within the multi-enzyme complex of PKS and NRPS can lead to a functional complex, which will synthesize a novel polyketide (Gokhale et al. 1999; McDaniel et al. 1999). Therefore, the recognition of the diversity of PKS and NRPS in the environment is important for future drug discovery and combinatorial biosynthesis efforts. Degenerate oligonucleotide primers have been designed to amplify the KS and A domains, which were most conserved in module PKS and NRPS, respectively (Moffitt and Neilan 2003; Neilan et al. 1999; Schirmer et al. 2005; Turgay and Marahiel 1994). They have been applied to study the distribution and diversity of PKS and NRPS genes in fungi (Nicholson et al. 2001), marine sponge-symbiotic microorganisms (Schirmer et al. 2005), cyanobacteria (Ehrenreich et al. 2005), and soil (Ginolhac et al. 2004). Up to date, no study on the diversity of genes for the synthesis of secondary metabolites in Antarctic environment has been reported, although many studies have revealed quite diverse and unique Antarctic microbial communities (Franzmann et al. 1996; Bowman et al. 2000; Sjöling and Cowan. 2003; Bowman and McCuaig. 2003; Jungblut et al. 2005). Here we present the first study of the diversity of PKS and NRPS genes in Antarctic sediment samples using two sets of degenerate oligonucleotide primers with the aim of understanding the potential of the Antarctic sediment microbial communities for production of secondary metabolites. The results revealed the great diversity of both PKS KS and NRPS A domains including many novel sequences in these Antarctic sediments.

Materials and methods

Samples

The samples ZSS and ZSN were collected from the coast near the China Zhongshan Station (69°22′S, 76°22′E) at the 22nd research voyage during November 2004–March 2005. The site ZSS was the sediment within the treated sewage effluent to the sea, while the ZSN site, 200 m away from ZSS site, was sediment of normal Antarctic seashore. The color of the ZSS sample was a little darker than that of ZSN sample, which was of sand color. The sediments between 10 and 15 cm below the surface were collected by sterile tools. The subsampling was carried out by discarding the surface on a clean bench immediately, then transferring it to the sterile falcon tubes and by keeping it in −20°C. The surface of the samples was discarded again on a clean bench after it was brought back to the lab. Only the central section was subjected to further analysis.

DNA extraction and amplification

The microbial community genomic DNA was extracted from ZSS and ZSN samples by combining chemical lysis and enzyme digestion, and then purifying by DNA-adsorbent resin (Zeng et al. 2005). The primers for the amplification of PKS KS domain were DKF (5′-GTGCCGGTNCCRTGNGYYTC-3′) and DKR (5′-GCGATGGAYCCNCARCARYG-3′) (Moffitt and Neilan 2001). The primers targeting NRPS A domain were MTF (5′-GCNGGYGGYGCNTAYGTNCC-3′) and MTR (5′-CCNCGDATYTTNACYTG-3′) (Neilan et al. 1999). The initial denaturation step at 94°C for 2 min was followed by 35 cycles of DNA denaturation at 94°C for 1 min, primer annealing at the corresponding annealing temperature for 1 min, and DNA strand extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. The annealing temperatures for the primer pair MTF/MTR and DKF/DKR were 50 and 51°C, respectively. Five replicate PCRs were

mixed together for each sample to reduce random biases within individual PCR reactions.

Cloning and RFLP

Amplified PKS and NRPS fragments were purified from 1% agarose and subsequently cloned into pTA2 (ToYoBo, Convenient TA cloning system) according to the manufacturer's instructions. One hundred clones were selected at random from each clone library of two samples for further analysis. The single colony was placed into 50 µl of double distillation water and treated by ultrasound at 59 Hz 54 W for 1 min. The preparations were centrifuged at 5,000*g* for 2 min, and the supernatant was used as a crude plasmid template for PCR. Inserts were amplified from the plasmid templates in 25 µl volumes, using standard T3 and T7 promoter primers. The initial denaturation step at 94°C for 2 min was followed by 30 cycles of 94°C for 1 min, 51°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min. To eliminate identical clones, each PCR product was digested with ten units of *Taq* I (ToYoBo) at 65°C for 2 h. After digestion, restriction fragment length polymorphism (RFLP) patterns were screened using 4% agarose gel electrophoresis. The clones with different digestion profiles were picked and sequenced.

Sequence analysis

The obtained KS domain (~700 bp) and A domain (~1,000 bp) sequences were analyzed by BlastX program in the NCBI databank (http://www.ncbi.nlm.nih.gov/BLAST/) to check the identity of AA sequence to known PKS and NRPS sequences in database. The comparison of the conserved motif of AA sequences was carried out by DNAMAN program. In the phylogenetic analysis, the alignment of deduced AA sequences was performed with Clustal X, and the unrooted neighbor-joining phylogenetic tree was constructed using MEGA verson 2.1 with the Poisson correction model for AA, complete deletion handling of gaps, and a bootstrap consisting of 1,000 replications.

Nucleotide sequence accession numbers

The AA sequences presented in this study have been deposited in the EMBL database under the accession numbers of AM492212-AM492237, AM492270-AM492273 (PKS KS domains), and AM492238-AM492269 (NRPS A domains).

Results

Analysis of sequences

One hundred and seventy-seven sequences were amplified from two PKS KS domain clone libraries constructed from ZSS and ZSN samples. Based on the comparison of RFLP profiles, 14 and 17 different clones were identified and sequenced from ZSS and ZSN samples, respectively. These sequences all showed below 93% identity between each other. From the NRPS A domain clone libraries, a total of 156 sequences were amplified, among which 13 and 18 clones with different RFLP profiles were identified from ZSS sample and ZSN sample, separately. All of the 62 sequences were analyzed at the AA level by BlastX. The diversities of both KS and A domains in the ZSN sample were different from that in the ZSS sample. To further explore their homology and novelty, phylogenies were constructed using the obtained PKS KS and NRPS A domains, and genes with high similarity in the BLAST searches.

Phylogenetic analysis of the detected PKS KS domains

All the obtained KS domains were below 80% similar to referenced sequences retrieved from the GenBank database at the AA level. The phylogenetic tree based on deduced AA sequences showed great diversity of KS domains (Fig. 1). The sequences isolated from ZSN and ZSS samples each tend to cluster in independent clades on the phylogenetic tree. Among the 31 obtained sequences, nine of them exhibited less than 58% identities to their closest matches in GenBank, and formed four distinct clades labeled as ZS1 to ZS4 (Fig. 1), except in ZS3, where the Antarctic sequences are mixed with non-Antarctic reference sequences. Three sequences (ZSS12, ZSS13, and ZSS14) were clustered in an independent clade labeled as ZS5 on the phylogenetic tree (Fig. 1), although they showed about 70% identities to KS domains from various bacteria. The low similarity prohibited the prediction of possible biosynthesis pathways and substrates by the alignment of KS domains to homologous fragments.

Type I KS domain AA sequences appeared to cluster phylogenetically into two functional groups. The first group represents KS domains, which use acyl-CoA's as their starter or extender unit and another distinct cluster is comprised of those sequences isolated from hybrid or mixed PKS/NRPS systems which condense AAs onto a polyketide extender unit (Ginolhac et al. 2004; Moffitt and Neilan 2003). In the type I bacterial ketosyntases, the obtained KS domains showed high homology with those from seven

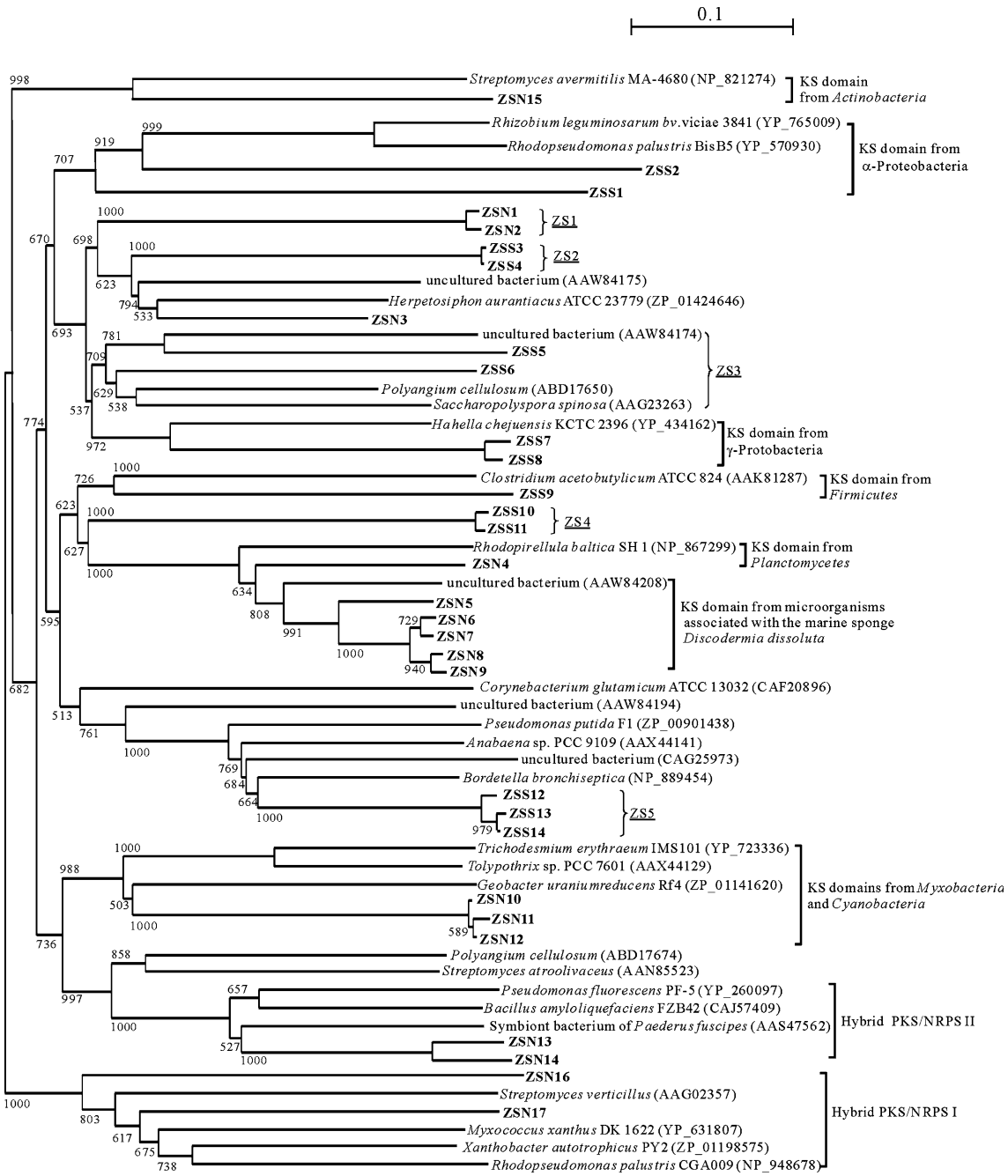

Fig. 1 Phylogenetic analysis of type I PKS KS domains from Antarctic sediment. Bootstrap values of >500, caculated from 1,000 bootstrap tress are indicated at the nodes. The clones in this study are in *boldface*. The *numbers* in the *brackets* are the GenBank accession numbers of the referenced AA sequences. The *scale bar* represents 0.1 AA substitution per position

bacterial groups, including *Cyanobacteria*, marine sponge-symbiotic microorganisms, α- and γ-*Proteobacteria*, *Firmicutes*, *Planctomycetes*, and *Actinobacteria* (Fig. 1). It was interesting that five KS domains obtained, all from the ZSN sample, exhibited closest similarity to that of the uncultured symbiotic bacteria from marine sponge *Discodermia dissolute*, with the highest AA similarity at 80% (Schirmer et al. 2005). They formed the largest branch on phylogenetic tree. The diverse bacteria harboring in this marine sponge have been found to be a rich source of potentially valuable natural products, such as cytotoxic, antibacterial, antifungal, antiviral, or anti-inflammatory compounds (Jimeno et al. 2004; Schirmer et al. 2005). Up to date, the *D. dissolute* has not been recorded in Antarctic. Our results indicated that Antarctic PKS modules with this type of KS domain are worth studying further.

Cyanobacteria are known as an important phylum with members containing PKS gene clusters which producing a functionally diverse class of biochemically synthesized compounds. In this study, KS domains of *Cyanobacteria* were not dominant in both ZSN and ZSS samples. The KS domains of *Myxobacteria* appeared to be phylogenetically similar to that of *Cyanobacteria*, indicating an evolutionary relationship between the enzymes of these two bacterial families. The sequences ZSN10, ZSN11, and ZSN12 were closely related to this cluster (Fig. 1), implying that they were amplified from cyanobacterium or myxobacterium in ZSN samples.

In a previous study, two patterns were used to identify KS domains belonging to hybrids between NRPS and PKS I and, more precisely, KS domains preceded by an NRPS, thus acting on a AA chain (Moffitt and Neilan 2003). Those two patterns were N(DE)KD at 22 AAs upstream from the cysteine active site in the KS domain and the VQTACSTS, which replaced the conserved pattern VDTACSSS of a typical KS domain (AA modification is shown with underline) (Fig. 2). In our study, two sequences (ZSN16 and ZSN17) were clustered within the hybrid PKS/NRPS I (Fig. 1), in which the referenced sequences had both N(DE)KD and VQTACSTS motifs. The sequence ZSN17 also had both these two patterns, showing 55% AA identity to a sequence from photosynthetic bacterium *Rhodopseudomonas palustris*, which contained McyE PKS and peptide synthetase (Larimer et al. 2004). However, the other sequence (ZSN16) had none of the patterns. Noticeably, two obtained KS domains (ZSN13 and ZSN14) showed about 68% identity to the reported hybrid NRPS/PKS sequences (Paulsen et al. 2005; Piel et al. 2004), but they formed an independent branch apart from hybrid PKS/NRPS I on the phylogenetic tree, labeled as hybrid PKS/NRPS II (Fig. 1). Compared with that of the hybrid PKS/NRPS I, the AA sequences of hybrid PKS/NRPS II did not possess the N(DE)KD motif, and the motif VQTACSTS was replaced by VETACSSS (Fig. 2). This implies that the two motifs reported previously were not unique to KS domains of hybrid PKS/NRPS enzyme complexes.

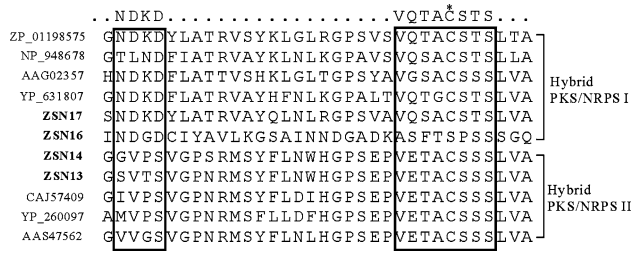

Fig. 2 Amino acid sequence alignment of the patterns for identification of hybrid PKS/NRPS enzyme complexes. The cysteine active site and the motifs N(DE)KD and VQTACSTS are marked by *asterisk* and *frame*, respectively. The sequences in this study are in *boldface*. The reference AA sequences in GenBank were derived from the following bacterial species as follows: ZP_01198575, *Xanthobacter autotrophicus* PY2; NP_948678, *Rhodopseudomonas palustris* CGA009; AAG02357, *Streptomyces verticillus*; YP_631807, *Myxococcus xanthus* DK 1622; CAJ57409, *Bacillus amyloliquefaciens* FZB42; YP_260097, *Pseudomonas fluorescens* PF-5; AAS47562, symbiont bacterium of *Paederus fuscipes*

Phylogenetic analysis of the detected NRPS A domain

Similar to the results of KS domain, the NRPS A domains from ZSN and ZSS samples formed tightly linked monophyletic clades on the phylogenetic tree, respectively (Fig. 3). Their closet matches were identified from diverse bacterial groups including *Cyanobacteria*, α-, β-, δ-, and γ-*Proteobacteria*, and *Firmicutes* (Fig. 3). They all showed less than 60% of AA sequence identity to their closet matches in GenBank.

Eight core motifs, A1 to A8 (A1, LKAGxAYVPID; A2, LAYxxYTSGTTGxPKG; A3, FDxS; A4, NxYGPTE; A5, GELxLxGxGLARGYW; A6, YKTGDQ; A7, GRxDxQVKIRGx; A8, NGKIDR) are highly conserved in the NRPS A domain which surrounds the active site where the substrates bind (Stachelhaus et al. 1999). All the A domains in this study contained these eight motifs except ZSN107, ZSN112, and ZSN113, which had a different sequence in A2 and A4 motifs. It implied that the obtained A domains shared cognate substrates and metabolism pathway, and generated the corresponding aminoacyl adenylate.

In contrast to the PKS KS domain phylogeny, most of the NRPS A domains fell into the *Cyanobacteria* and *Myxococcales* cluster, which was the largest branch on the phylogenetic tree (Fig. 3). Eight sequences of them showed high similarity to the A domains from genus *Nostoc*, which was widespread in aquatic environments and received most study among the *Cyanobacteria* group. By comparing with sequenced cyanobacterial genomes, *N. punctiforme* ATCC29133 has been found to possess the greatest number of NRPS genes (17 genes) and PKS genes (ten genes) in its annotation (Ehrenreich et al. 2005). In the phylogeny of the A domains, many identified fragments cluster with proteins described in the literature (Fig. 3), including NosA, which is involved in the nostopeptolide synthesis in *Nostoc* sp. strain GSV224 (Hoffmann et al. 2003); NcpB, which is involved in 4-methylproline synthesis in *Nostoc* sp. strain ATCC 53789 (Luesch et al. 2003); McyA, which is involved in microcystin synthsis in *Microcystis aeruginosa* (Nishizawa et al. 1999), and JamO, which, is involved in a new mixed polyketide-peptide neurotoxin from the marine Cyanobacterium *Lyngbya majuscule* (Edwards et al. 2004).

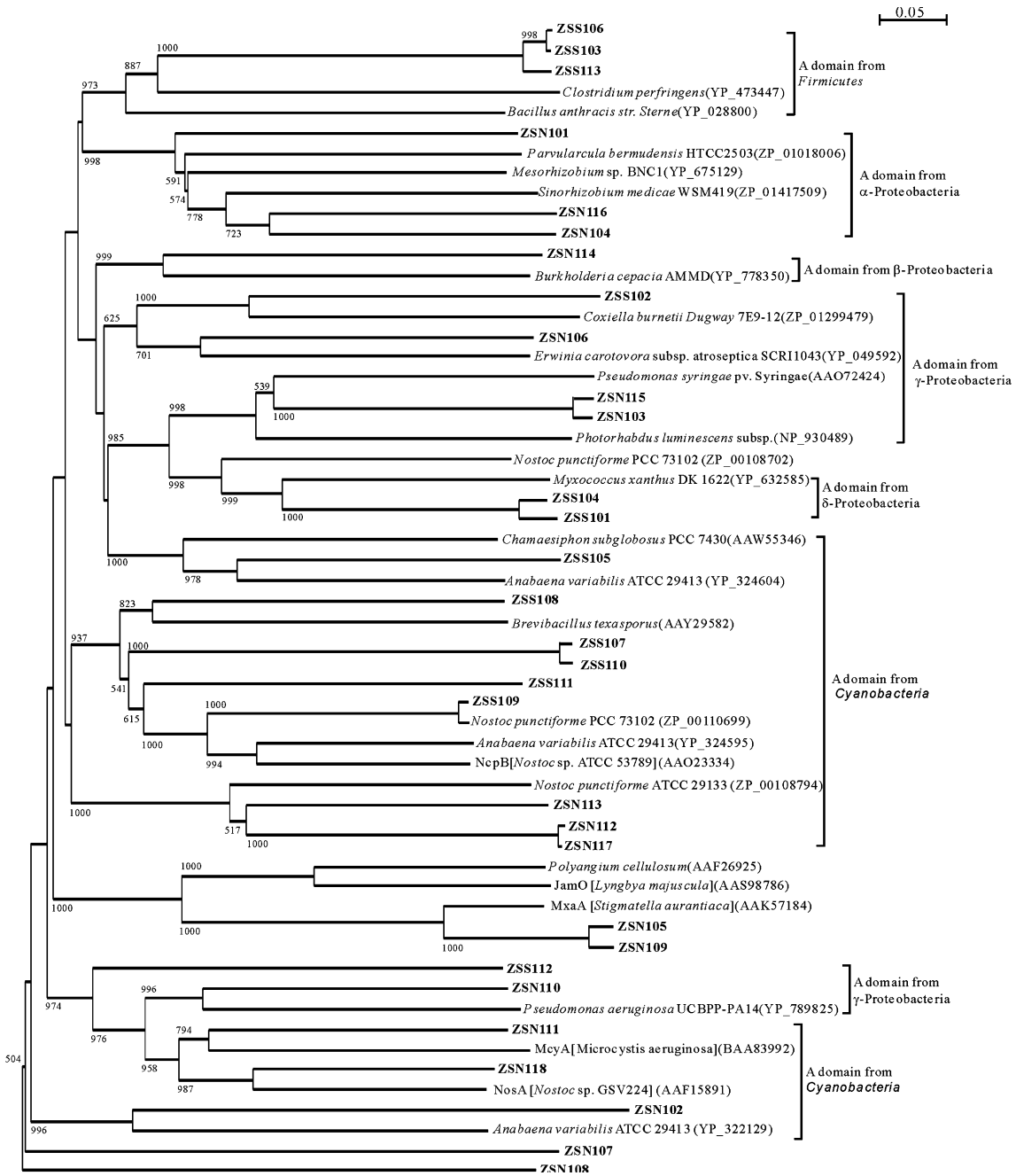

Fig. 3 Phylogenetic analysis of NRPS A domains from Antarctic sediment. Bootstrap values of >500 caculated from 1,000 bootstrap tress are indicated at the *nodes*. The clones in this study are in *boldface*. The *numbers* in the *brackets* are the GenBank accession numbers of the referenced AA sequences. The *scale bar* represents 0.05 AA substitution per position

The A domains from *Proteobacteria* were also found to be prevalent in this study, especially in ZSN sample. Although three sequences (ZSS103, ZSS106, and ZSS113) formed a distinct clade due to low similarities to the bacitracin synthetase of *Clostridium perfringens* (less than 42%), they still clustered within *Firmicutes*, indicating a phylogenetically relationship of the functionality of the NRPS domain.

Discussion

Sequence analysis of the active site, and phylogenetic reconstruction provided us with an insight into the activity of the KS and A domains within environmental samples. In this study, degenerate oligonucleotide primers were applied to amplify the KS domain of type I PKS and A domain of NRPS modules. These primers were designed by the

alignment of KS and A domains in known gene clusters of bacteria, including *Cyanobacteria*, *Actinobacteria*, and *Firmicutes* (Moffitt and Neilan 2001, 2003; Neilan et al. 1999). However, a much broader distribution of KS and A domains was found in our samples, including those clustering with α-, β-, γ-, δ- *Proteobacteria*, *Planctomycetes*, *Cyanobacteria*, *Actinobacteria*, *Firmicutes*, and some uncultured symbiotic bacteria associated with a marine sponge, indicating that the primers were effective in the studies of gene diversity in environmental samples. Most of the identified fragments exhibited low similarity to literature sequences in GenBank (KS, less than 80%; A, less than 60% BLASTX identities with their closest neighbors) and were not identical to each other. Phylogenies were constructed to study the structural and functional relatedness between these identified genes to other well-described genes in GenBank. Corresponding to the low-sequence similarity, nearly half of obtained KS domains formed phylogenetically distinct clades, indicating that the organisms containing these genes have not been isolated or analyzed for their PKS gene pathways yet. Furthermore, a different branch of hybrid PKS/NRPS enzyme complexes, labeled as hybrid PKS/NRPS II, was identified on the phylogenetic tree (Fig. 1). The sequences in this branch had no typical motifs, which were considered as unique to hybrid PKS/NRPS genes previously studied (Moffitt and Neilan 2003). All these results indicated that the diversity and novelty of genes for the synthesis of bacterial natural compounds in Antarctic environment was greater than expected, and this suggests that corresponding natural products may also be diverse and novel.

Many *Actinobacteria* contain PKS and NRPS gene clusters and this group of bacteria has long been recognized as an important source of bioactive molecules (Moore et al. 2005). However in our Antarctic samples, only one PKS KS domain (ZSN15) showed 52% identity to *Streptomyces avermitilis*, and none of the NRPS A domains was affiliated with the *Actinobacteria* group. In our denaturing gradient gel electrophoresis analysis, no 16S rRNA sequence affiliating with *Actinobacteria* was identified from both ZSN and ZSS samples (Lin and Zeng 2007). Only one sequence belonging to *Actinobacteria* was recovered from 16S rDNA clone library of ZSN sample, and none was found from the library of ZSS sample (data not shown). It could be deduced that *Actinobacteria* was not the dominant group producing bioactive compounds in the Antarctic samples studied.

Many organisms are known to produce bioactive secondary metabolites in response to environmental pressures (Baker et al. 1995). Study on the genetic potential for secondary metabolite production in stromatolite communities also indicated that the microorganisms thriving in the hypersaline reaches of Shark Bay might have developed similar defence mechanisms in response to their extreme habitat (Burns et al. 2005). But a satisfying hypothesis explaining the relationship between microbial secondary metabolite production and environmental parameters has not been elaborated yet. In this study, the diversities of both KS and A domains in ZSN sample were quite different from those in ZSS sample. The number of both the KS and A domain clones with different RFLP profile retrieved from the ZSN sample were greater than the number of these from the ZSS sample. The content of the total organic carbon in ZSS and ZSN samples were 2.866 and 0.840 mg/g, respectively, estimated by the burning oxidation-non-dispersive infrared method (TOC-V_{CPN}, Shimadzu, Japan). Organic compounds have important impacts on the microbial diversity and abundance, and the underlying molecular diversity in environment. Therefore, the quality and quantity of nutrients could be deduced as one of the important factors resulting in the differences between the diversity of KS and A domains in ZSN and ZSS samples.

We obtained a detailed picture of the diversity of genes for biosynthesis of polyketides and nonribosomal peptides in two Antarctic environmental samples. None of the obtained sequences showed higher than 80% identity at the AA level to sequences described in GenBank (as of January 2007), implying their chemical and functional novelty. The pathway and substrate specificities of these unknown sequences were difficult to deduce because of the low similarity, but are distinct enough to deserve further study. We have confirmed that potentially very diverse biosynthesis pathways and novel natural compounds might be present in Antarctic environment, and future studies on drug discovery could start from Antarctic microbial communities.

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