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ORIGINAL ARTICLE

The Screening of Antimicrobial Bacteria with Diverse Novel Nonribosomal Peptide Synthetase (NRPS) Genes from South China Sea Sponges

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Abstract Nonribosomal peptide synthetase (NRPS) adenylation (A) domain genes were investigated by polymerase chain reaction for 109 bacteria isolated from four South China Sea sponges, *Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis*. Meanwhile, the antimicrobial bioassay of bacteria with NRPS genes were carried out to confirm the screening of NRPS genes. Fifteen bacteria were found to contain NRPS genes and grouped into two phyla Firmicutes (13 of 15) and Proteobacteria (two of 15) according to 16S rDNA sequences. Based on the phylogenetic analysis of the conserved A domain amino acid sequences, most of the NRPS fragments (11 of 15) showed below 70% similarity to their closest relatives suggesting the novelty of these NRPS genes. All of the 15 bacteria with NRPS genes have antimicrobial activities, with most of them exhibiting activity against multiple indicators including fungi and gram-positive and gram-negative bacteria. The different antimicrobial spectra indicate the chemical diversity of biologically active metabolites of sponge-associated bacteria and the possible role of bacterial symbionts in the host's antimicrobial chemical defense. Phylogenetic analysis based on the representative NRPS genes shows high diversity of marine NRPS genes. The combined molecular technique and bioassay strategy will be useful to obtain sponge-associated bacteria with the potential to synthesize bioactive compounds.

Keywords Sponge · Bacteria · Nonribosomal peptide synthetase (NRPS) · PCR · Phylogenetic analysis · Antimicrobial activity

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Introduction

Since the 1950s, many structurally diverse natural products with astounding bioactivities have been discovered from marine organisms (Blunt et al. 2007, 2008). Many researchers suggest that microbial symbionts are at least involved in the biosynthesis of their hosts' metabolites or are in fact the true origin of some metabolites (Fortman and Sherman 2005; König et al. 2006). So, marine microorganisms, especially those associated with marine organisms, namely, marine microbial symbionts, have become one of the research hotspots of marine microbiology and marine natural products (Schmidt 2005; Salomon et al. 2004).

Because of the tremendous importance of nonribosomal peptides for modern medicine, the investigation of their biosynthesis is of great value (Schwarzer et al. 2003). Producers of nonribosomal peptides are mostly soil-inhabiting microorganisms, such as *Actinomycetes* and *Bacilli* (Cheng et al. 2002; Horwood et al. 2004). But, recent studies show that marine microorganisms have also emerged as important sources for nonribosomal peptide metabolites (Blunt et al. 2007, 2008). Nonribosomal peptides are often quite complex in structure and difficult to synthesize. By screening microorganisms with the potential to biosynthesize nonribosomal peptides, non-

Table 1 Primers for PCR screening of NRPS A domain genes

Primer	Sequence(5′–3′)	Reference
MTR	5′-GCNGGYGGYGCNTAYGTNCC-3′	Vizcaino et al. 2005
MTF	5′-CCNCGDATYTTNACYTG-3′	
NS1	5′-CAACCCCTATGCCTT TTGAA-3′	Schmidt et al. 2004
NS2	5′-TAAACAACCCATGCTCCACA-3′	
NP1	5′-CCTAATTCAATACGAAAACCACGAADYTTNAYYTG-3′	Schmidt et al. 2004
NP2	5′-TGTATGTTATTTATACTTCTGGTTCTACTGGTMRNCCANARG G-3′	

ribosomal peptides can be produced by microbial large-scale fermentation to solve the resource problem of marine natural products.

The nonribosomal peptide synthetase (NRPS) gene clusters have been found to synthesize a diverse array of biologically active nonribosomal peptides, including antibiotics (e.g., penicillin and vancomycin; Cane et al. 1998), anti-inflamatorials and immunosuppressants (e.g., cyclosporin A) (Cane and Walsh 1999), toxins (Cane et al. 1998), and siderophores (Crosa and Walsh 2002). Meanwhile, the direct manipulation of related gene clusters would allow the generation of new drugs (Fortman and Sherman 2005). A NRPS module usually contains an adenylation (A) domain, a peptidyl carrier protein domain, and a condensation domain. Nonribosomal peptide synthesis usually ends with a thioesterase domain, which catalyzes the termination of chain elongation (Schwarzer et al. 2003). As for revealing NRPS gene diversity, the related degenerate oligonucleotide primers have been designed to amplify the most conserved domain of NRPS such as A domain (Schirmer et al. 2005). By this approach, the distribution and diversity of NRPS genes in cyanobacteria (Ehrenreich et al. 2005), fungi (Johnson et al. 2007), and bacteria (Zhao et al. 2008) have been revealed.

Though culture-independent studies have revealed some information about NRPS genes from marine environment (Schirmer et al. 2005), it is hard to locate the exact biological origin. So, culture-dependent methods have been applied in the investigation of NRPS genes from marine environment, and some of the culturable isolates have been shown to produce bioactive compounds (Jiang et al. 2007; Pathom-Aree et al. 2006; Zhu et al. 2007).

Sponges (Porifera) harbor abundant and diverse microorganisms (Li et al. 2006, 2007a), exceeding that of seawater by two to three orders of magnitude (Hentschel et al. 2006), and are known to be the sources of various marine bioactive metabolites including unusual nonribosomal peptide synthetase and polyketide synthase (PKS) products (Phuwapraisirisan et al. 2005). Sponge-associated bacteria are important resources of marine natural products (Hentschel et al. 2006) and have been proved to synthesize polyketides and nonribosomal peptide compounds (Grozdanov and Hentschel 2007; Schmidt 2005; Salomon et al. 2004). But at present, the microbial NRPS genes associated with sponges are rarely investigated (Jiang et al. 2007; Piel et al. 2004; Schirmer et al. 2005). According to Blunt et al. (2007), the China Sea has become an important source region

Table 2 Similarity to the closest relatives in GenBank of 16S rDNA sequences of isolates from sponges

Strain	Accession no.	Closest relative and its accession number	% Identity
A05	DQ091002	*Alcaligenes* sp. FP3-2 (EU293345)	100
A11	DQ274113	*Alcaligenes* sp. L981 (AY371437)	98
A75	DQ091004	*Staphylococcus lentus* SSH39 (AB219154)	100
B04	DQ277981	*Bacillus licheniformis* 3EC4C4 (EU304953)	100
B05	DQ277982	*Bacillus* sp. A5 (EU363684)	100
B27	EF370049	*Bacillus subtilis* MO2 (AY553095)	100
B31	EU384285	*Bacillus* sp. Y01 (DQ531607)	100
B87	EU384286	*Bacillus* sp. KYJ963 (U49080)	100
B99	EU384287	*Bacillus* sp. gf-5 (AB219154)	100
B105	EU384288	*Bacillus flexus strain* XJU-4 (EF433402)	99
B114	EF370052	*Bacillus subtilis* CGMCC1869 (EF159949)	100
B131	EF370053	*Bacillus subtilis* BZ15 (AY162133)	100
B144	EF370054	*Bacillus subtilis* AU25 (EF032688)	99
B145	EF370055	*Bacillus subtilis* WL-6 (DQ198162)	100
C130	DQ091013	*Bacillus* sp. B75 Ydz-zz (EU368769)	100

of marine natural products since 2001, but the investigation of microbial NRPS genes associated with marine organisms in the China Sea has been rarely found (Jiang et al. 2007). So, it is therefore important to investigate the NRPS genes of China Sea sponge-associated microorganisms.

In this study, the NRPS genes of the cultivable bacteria associated with four South China Sea sponges were revealed by the conserved A domain polymerase chain reaction (PCR) and phylogenetic analysis. Meanwhile, the antimicrobial potential of the bacteria with NRPS genes were evaluated by bioassay. In addition, based on the representative A domains of NRPS gene clusters, the diversity of the present marine microbial NRPS genes are discussed.

Materials and Methods

Sponge-Associated Bacterial Isolation

Four South China Sea sponges reported as (Li et al. 2006), *Stelletta tenuis*, *Halichondria rugosa*, *Dysidea avara*, and *Craniella australiensis* were used for bacterial isolation by the method of Li et al. (2007b). The medium used for bacterial

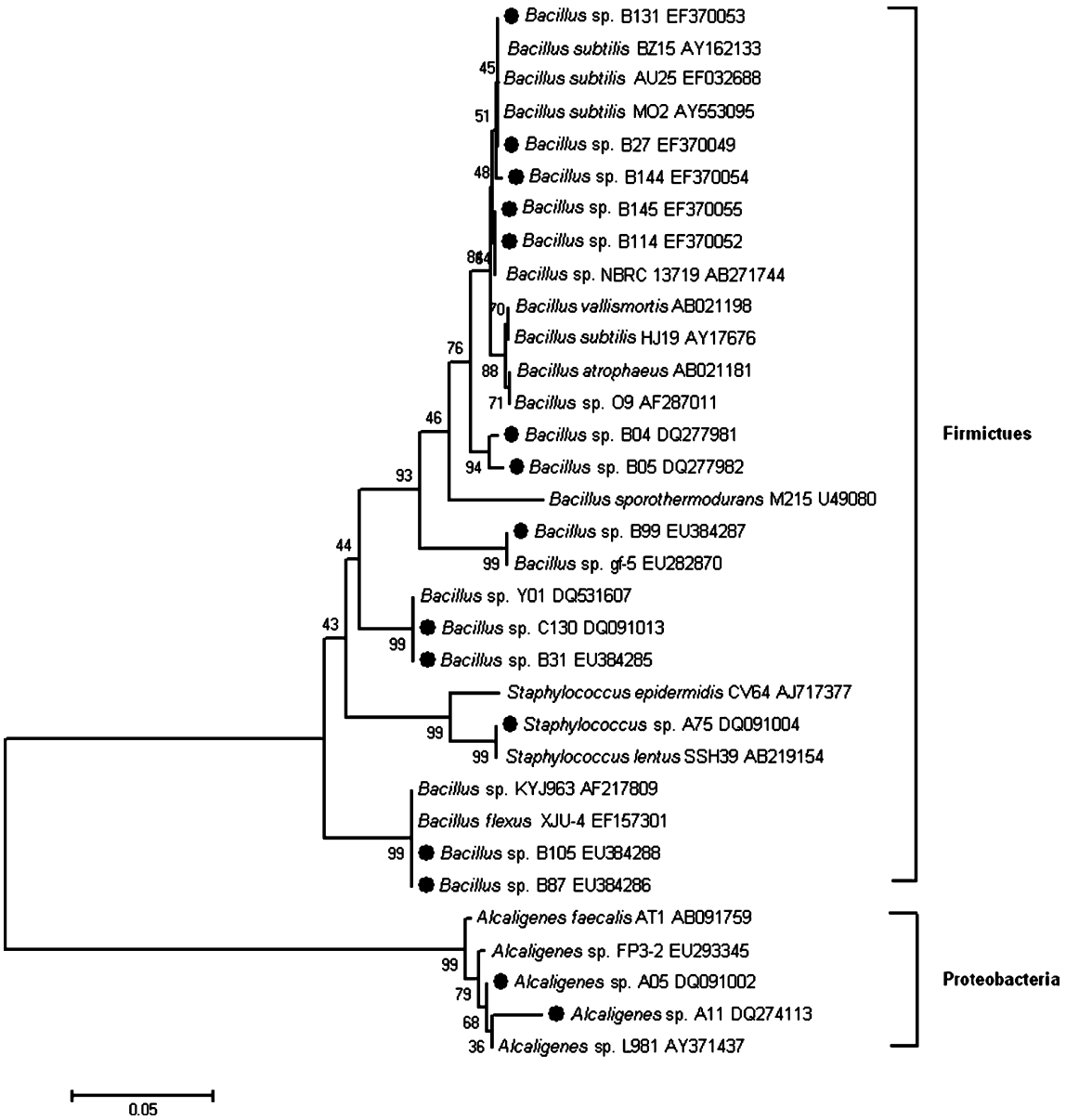

Fig. 1 Neighbor–joining phylogenetic tree based on approximately 1,500 bp 16S rDNA sequences of the isolates from sponges (*with dot in front*) and reference sequences. Percentage bootstrap values of neighbor-joining analysis from 1,000 resamplings are indicated at *nodes*. The *scale bar* represents 0.05 nucleotide substitutions per site

isolation contained 10 g/l peptone, 5 g/l beef extract, and artificial sea water (ASW) containing 26.518 g/l NaCl, 2.447 g/l $MgCl_2$, 3.305 g/l $MgSO_4$, 1.141 g/l $CaCl_2$, 0.725 g/l KCl, 0.202 g/l $NaHCO_3$, and 0.083 g/l NaBr (Li and Liu 2006). Nystatin (40 mg/ml) was used to prevent fungal growth, and pH was adjusted to 7.2–7.4. Agar (1.5%) was added in the above medium to prepare agar plates. The inoculated plates were incubated for 2–3 days at 28°C.

Genomic DNA Extraction

Bacteria were collected by centrifugation at 10,000 rpm for 10 min at 4°C and suspended in 100 μl of artificial seawater, and 150 μl of cell-break buffer (50 mM Tris, 5 mM ethylenediamine tetraacetic acid, 1% sodium dodecyl sulfate, pH 11.5) and 150 μl of proteinase K reaction buffer (50 mM Tris, 15 mM $CaCl_2$, pH 3.0) were added. After incubation with proteinase K (10 mg/ml) at 55°C for 30 min, genomic DNA was extracted in order with tris-phenol (pH 8.0), tris-phenol/chloroform/isoamyl alcohol (25:24:1), and chloroform/isoamyl alcohol (24:1) and precipitated by absolute isopropyl alcohol. RNA was removed by RNase (10 mg/ml, Sigma). The genomic DNA was stored at −20°C before PCR amplification.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Only the bacteria with NRPS genes and antimicrobial activity were identified by 16S rRNA gene sequencing. Amplification of 16S rDNA was performed in Master Cycler Gradient (HYBAID, Middlesex, UK) with primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-CTACGGCTAC CTTGTTACGA-3′) according to the protocol as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min and a final extension of 10 min at 72°C. PCR products of approximately 1,500 bp were purified by electrophoresis in 1.5% (wt/vol) agarose gel and recovered using a gel purification kit (Shenergy Biocolor Bioscience & Technology Company, Shanghai, China). The PCR products were then ligated into pUCm-T vector and transformed into $CaCl_2$-competent *Escherichia coli* DH5a. The positive recombinants were screened on 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside-isopropyl-β-D-thiogalactopyranoside-ampicillin-tetracycline indicator plates by color-based recombinant selection. Positive clones were identified by PCR amplification using pUCm-T vector primer pairs T7 (5′-TAATACGACTCACTATAGGG-3′) and M13 (5′-CAGGAAACAGCTATGACC-3′) with the same procedure described above using 55°C as the T_m temperature. A nearly full sequence of 16S rDNA was determined by ABI 3730 automatic capillary DNA sequencers (AGRF, Brisbane, Australia). 16S rDNA sequences were matched to sequences in GenBank database using the nucleotide BLAST with default parameters within National Center for Biotechnology Information (NCBI). Sequences were aligned against reference sequences by CLUSTAL X, and then the aligned dataset was used as input for phylogenetic analysis programs. Phylogenetic analysis was performed with program Molecular Evolutionary Genetics Analysis, version 3.1 (MEGA 3.1; Kumar et al. 2004). Tree topology was evaluated by bootstrap analyses based on 1,000 replicates, and phylogenetic tree was inferred using the neighbor-joining method.

The obtained 16S rRNA gene sequences were deposited in GenBank with accession numbers EU384285–EU384288, EF370049, EF370052, EF370053, EF370054, EF370055, DQ274113, DQ091004, DQ091002, DQ277981, DQ277981, and DQ091013.

Table 3 Similarity to the closest relatives in GenBank of NRPS amino acid sequences of isolates from sponges

Sequence	NRPS Accession No.	Closest NRPS relative and its accession number	% Identity
NA05	EU399161	*Staphylococcus equorum* WS2733 (AAG16975)	64
NA11	EU399162	*Staphylococcus equorum* WS2733 (AAG16975)	63
NA75	EU399163	*Staphylococcus equorum* WS2733 (AAG16975)	69
NB04	EU399164	*Bacillus licheniformis* ATCC 10716 (AAD04757)	96
NB05	EU399165	*Bacillus subtilis* str. 168(NP_389602)	93
NB27	EU399166	BmyB *Paenibacillus larvae* BRL-230010(ZP_02327133)	51
NB31	EU399167	MycC *Bacillus subtilis* ATCC6633 (AAF08797)	52
NB87	EU399168	*Clostridium cellulolyticum* H10 (ZP_01573794)	48
NB99	EU399169	*Bacillus pumilus* SAFR-032 (YP_001485577)	84
NB105	EU399170	*Staphylococcus equorum* WS2733 (AAG16975)	70
NB114	EU399171	*Bacillus pumilus* SAFR-032 (YP_001485577)	51
NB131	EU399172	*Hahella chejuensis* KCTC 2396 (YP_433290)	44
NB144	EU399173	*Bacillus weihenstephanensis* KBAB4 (ZP_01182549)	47
NB145	EU399174	*Clostridium cellulolyticum* H10 (ZP_01573794)	49
NC130	EU399175	*Staphylococcus equorum* WS2733 (AAG16975)	63

NRPS Genes Screening and Phylogenetic Analysis

A series of primers listed in Table 1 were used to amplify the conserved A domain of nonribosomal peptide synthetase gene cluster (Schmidt et al. 2004; Vizcaino et al. 2005). PCR was performed using the following amplification parameters: initial denaturation at 94°C for 5 min, then 35 cycles of 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 10 min. The products were assessed by gel electrophoresis for the presence of 1,000 bp size amplicons, and the correct size amplicons were then cloned into pUCmT vector. The positive clones were proved by PCR using pUCm-T vector primer pairs T7 (5′-TAATACGACTCACTATAGGG-3′) and M13 (5′-CAGGAAACAGCTATGACC-3′) with the same procedure described above. The sequences of inserts were determined by ABI 3730 automatic capillary DNA sequencers (AGRF, Brisbane, Australia) using T7 or M13 primers.

The deduced amino acid sequences of NRPS genes were used as queries to search the related proteins in the nr protein database at NCBI using the BLASTP algorithm with default parameters. NRPS phylogenetic tree was constructed using the multiple sequence alignment tool in

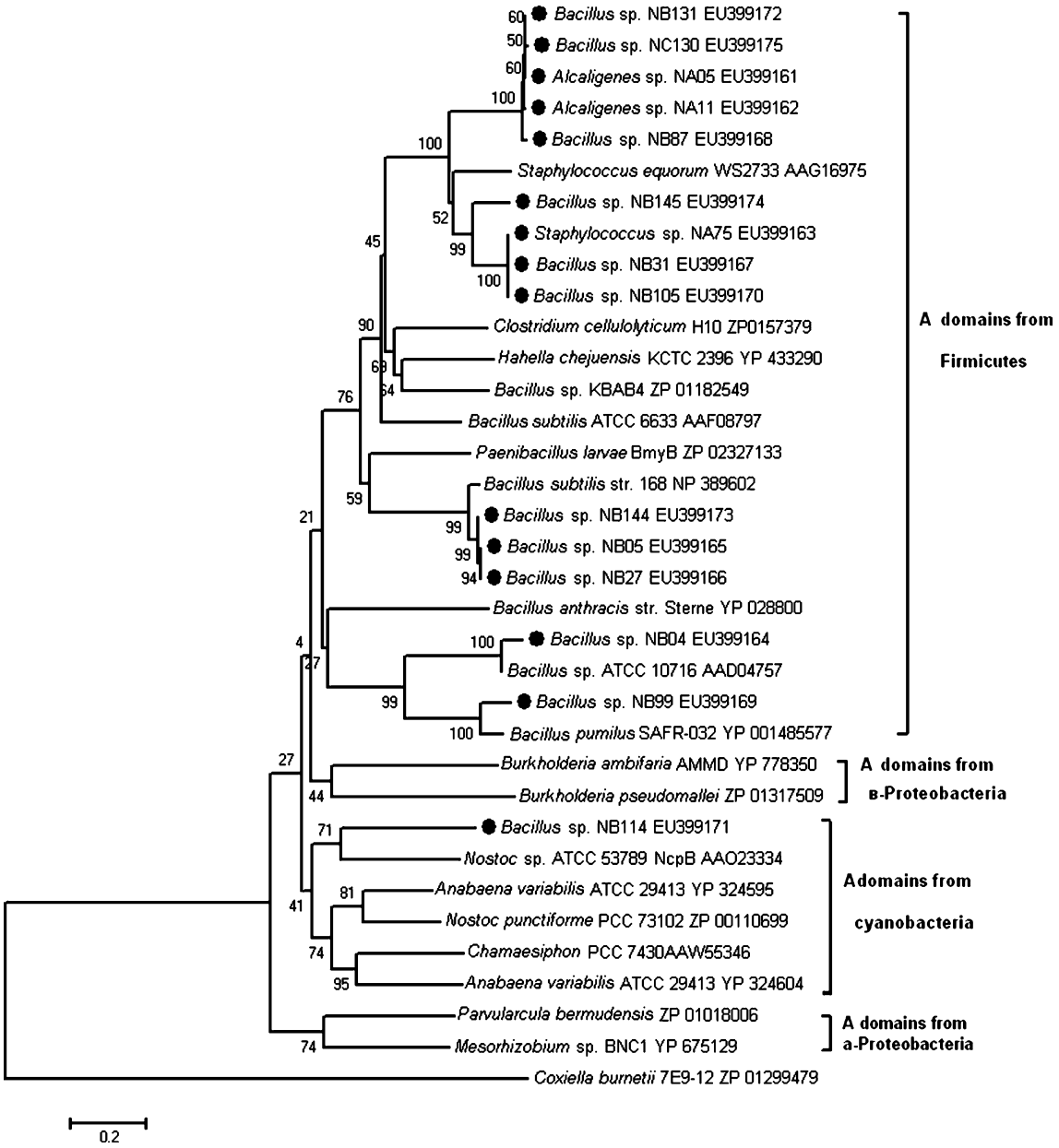

Fig. 2 Neighbor-joining phylogenetic tree from analysis of A domain amino acid sequences of isolates from sponges (*with dot in front*) and reference sequences of known NRPS sequences. Percentage bootstrap values of neighbor-joining analysis from 1,000 resamplings are indicated at *nodes*. The *scale bar* represents 0.2 AA substitutions per site

CLUSTAL X and MEGA package. Neighbor-joining method was adopted with 1,000 bootstrap resembling.

The obtained NRPS gene sequences were deposited into GenBank under the accession numbers EU399161 to EU399175.

Bioassay of Antimicrobial Activity

Antimicrobial bioassay was carried out by paper-disc plate method using six indicators including *E. coli* (AS 1.3373), *Staphylococcus aureus* (AS 1.2465), *Pseudomonas fluorescens* (AS 1.55), *Bacillus subtilis* (AS 1.3343), *Candida albicans* (AS 2.0086), and *Aspergillus niger* (AS 3.5487) from the Chinese Biodiversity Information Center, Chinese Academy of Sciences. Before bioassay, the isolated bacterium was cultured in medium containing 10 g/l peptone, 5 g/l beef extract prepared with artificial sea water (Li and Liu 2006) at 37°C for 24 h. Microbial indicators, except for *A. niger*, were firstly cultured in the same medium above using distilled water instead of ASW at 37°C for 18–24 h and

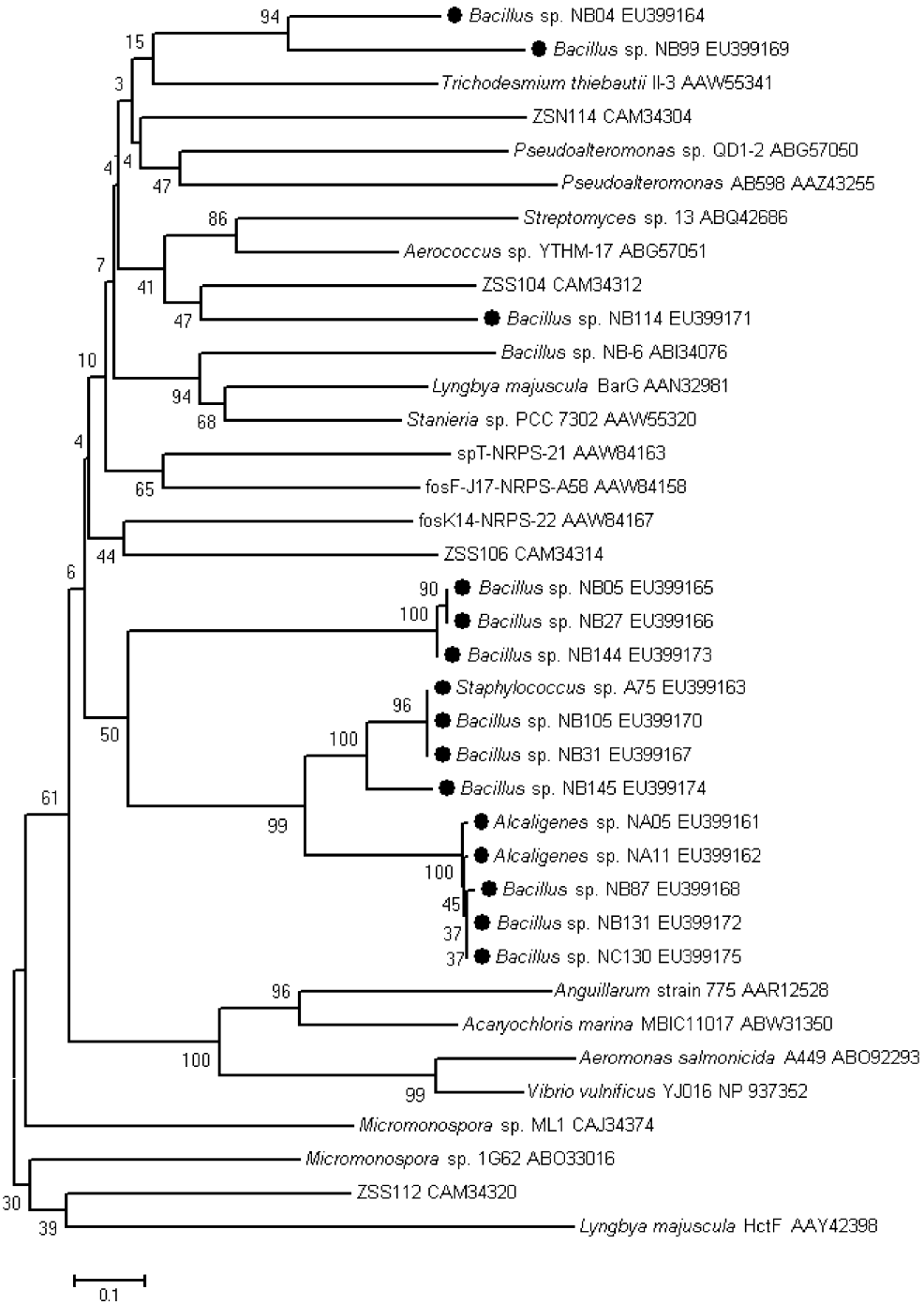

Fig. 3 Neighbor-joining phylogenetic tree from analysis of A domain amino acid sequences of isolates from sponges (*with dot in front*) and reference sequences of marine-origin A domains of NRPSs. Percentage bootstrap values of neighbor-joining analysis from 1,000 resamplings are indicated at *nodes*. The *scale bar* represents 0.1 AA substitutions per site

then spread on plates with the same medium containing 2% agar. As for indicator *A. niger*, it was firstly cultured in medium containing 30 g/l sucrose, 2 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l KCl, and 0.01 g/l $FeSO_4$ at 28°C for 7 days and then spread on plates with the same medium containing 2% agar. Filter paper disks (5 mm in diameter) were wetted with 0.04 ml isolate cultures and put on top of the indicator plate. After co-incubation at 28°C for 3–4 days for indicator *A. niger* and 37°C for 1 day for the other five indicators, the diameter (mm) of the clear inhibition zone produced around each paper disk was measured. All the tests were repeated three times with one single test in replicates. The final value of zone diameter was an average of six results.

Results and Discussion

Screening of Sponge-Associated Bacteria with NRPS Genes

Among the 109 isolates from four South China Sea sponges, *S. tenuis*, *H. rugosa*, *D. avara*, and *C. australiensis*, fifteen isolates were found to be positive for NRPS genes by PCR screening of the A domain. According to the BLAST and phylogenetic analysis based on 16S rRNA gene sequences (approximately 1,500 bp) of the 15 isolates and the known sequences in GenBank database (Table 2 and Fig. 1), all the 15 bacteria show high similarity (>98%) to the relatives in GenBank and are grouped into two phyla Firmicutes (13 of 15) and Proteobacteria (two of 15). In our previous study (Li et al. 2006), Proteobacteria appeared to form the majority of the in vivo bacteria in these sponges. Here, only two isolates A05 and A11 are grouped in the phylum Proteobacteria. Isolate A05 matches a phenol degradation strain *Alcaligenes* sp. FP3–2, and A11's relative is a deep-water marine invertebrate-derived Betaproteobacteria species *Alcaligenes* sp. L981 (AY371437). In the Firmicutes group, 12 isolates were identified as *Bacillus* except for *Staphylococcus* sp. A75. *Bacillus* is a well-known producer of metabolites with antimicrobial or generally cytotoxic properties. Though the genus *Bacillus* is regarded to be of terrestrial origin, many have been isolated from marine benthos such as soft corals (Kapley et al. 2007) and marine sponges (Prokofeva et al. 1999; Hentschel et al. 2001). Similarly, abundant *Bacillus* were isolated from South China Sea sponges in this study. Based on the biochemical tests, all these isolates grow well on media containing 2–6% NaCl (data not shown).

Diversity of NRPS Genes in Sponge-Associated Bacteria

The obtained 15 A domain gene sequences of NRPS were submitted to GenBank and analyzed by BLAST based on the deduced amino acid sequences. Most of the NRPS fragments (11 of 15) show below 70% similarity to their closest neighbors in GenBank at the AA level except for NB04, NB05, NB99, and NB105 (Table 3), suggesting the novelty of the NRPS genes in these South China Sea sponge-associated bacteria. Interestingly, only five NRPS genes match that of *Bacillus*, which is different from the bacterial identification (Table 2). According to Fig. 2, the obtained A domains are mainly clustered within the Firmicutes (14 of 15) and Cyanobacteria (one of 15)-derived NRPS groups. Some of the relatives are involved in

Table 4 Antimicrobial activities of the isolated bacteria with A domain genes from sponges

Strain	Indicator Microorganisms					
	S. aureus	*A. niger*	*C.albican*	*P. fluorescens*	*B. subtilis*	*E. coli*
A05	−	−	+++	−	−	++
A11	++	−	++	−	+	+
A75	++	++	+	+	++	−
B04	+	++	−	−	−	−
B05	+++	−	−	−	+	−
B27	++	+++	−	+	−	−
B31	+	+	−	−	+	−
B87	+	−	−	−	−	−
B99	+	−	−	++	−	−
B105	+	−	−	−	+	−
B114	+++	+	−	+++	−	+
B131	+	−	−	+	−	−
B144	+	+++	−	++	−	−
B145	+	+	−	++	−	−
C130	−	−	−	++	−	−

(−) no activity, (+) weak activity (5–10 mm halo), (++) good activity (10–15 mm halo), (+++) excellent activity (15–20 mm halo)

the biosynthesis of peptides, for example, the NRPS fragment of *Bacillus pumilus* SAFR-032 (YP001485577) with high similarity (84%) to NB99 is a non-ribosomal peptide synthetase subunit. MycC from *B. subtilis* ATCC6633 (AAF08797), the closest relative of NB31, is one open reading frame of the mycosubtilin synthetase gene cluster with strong homologies to the family of peptide synthetases. Meanwhile, *B. subtilis* ATCC6633 has been identified as a producer of mycosubtilin, a potent antifungal peptide antibiotic (Duitman et al. 1999). *Bacillus licheniformis* ATCC 10716 (AAD04757), with 96% similarity to NB04, can produce surface-active lichenysins with antibiotic properties (Konz et al. 1999). Several strains of *B. licheniformis* have been described as producers of lichenysins, which are widely used in the industry as enzyme and drugs (Rey et al. 2004). So, the similar function of the detected NRPS genes to their relatives in the biosynthesis of peptides is suggested to some extent.

Compared with PKS genes, few marine NRPSs genes have been revealed (Fortman and Sherman 2005; Schirmer et al. 2005). Based on the reported representative A domains of NRPS gene clusters, Fig. 3 was constructed to illustrate the relationship and diversity of marine microbial NRPS genes in order to have an overall understanding of the marine microbial NRPS genes identified. According to Fig. 3, 14 of 15 of the NRPS genes from the bacteria associated with South China Sea sponges cluster together forming two groups, which means that these NRPS genes are different from the other marine NRPS genes. Twelve NRPS genes grouped together show high similarity to three NRPS relatives of sponge-associated bacteria, A domain fragments AAW84167, AAW84163, and AAW84158 from the uncultured bacteria of sponges (Schirmer et al. 2005), suggesting the similar functions of these NRPS genes of bacteria associated with South China Sea sponges to that of the NRPS genes from other sponge-associated bacteria to some extent. Meanwhile, the closest NRPS relatives of the other three NRPS genes in this study are from cyanobacteria and uncultured bacteria. NRPS genes of NB04 and NB99 cluster with *Trichodesmium thiebautii* II-3 (AAW55341) from the marine cyanobacterial cultures (Ehrenreich et al. 2005). NRPS of NB114 clusters with CAM34312 from an uncultured bacterium isolated from the Antarctic sediment (Zhao et al. 2008); additionally, CAM34304, CAM34320, CAM34314 were also detected in this uncultured bacterium.

Besides the NRPS genes mentioned above, in Fig. 3, ABO92293 is from the draft genome sequence of *Aeromonas salmonicida* A449 isolated from numerous fish (Nash et al. 2006). *Pseudonocardia* sp. AB598 (AAZ43255) is from deep-sea sediment (10,898 m) in the Mariana Trench (Pathom-Aree et al. 2006). *Acaryochloris marina* MBIC11017 (ABW31350) is from a chlorophyll *d*-producing cyanobacterium (Swingley et al. 2008). *Stanieria* sp. PCC 7302 (AAW55320) is from marine cyanobacterial cultures (Ehrenreich et al. 2005). *Bacillus* sp. NB-6 (ABI34076) is from marine bacterium in the coastal area of China (Zhu et al. 2007). HctF (AAY42398) is one fragment of hectochlorin biosynthetic gene cluster from marine cyanobacterium (Ramaswamy et al. 2007). BarG (AAN32981) is a fragment of the barbamide biosynthetic gene cluster of mixed PKS-nonribosomal peptide synthetase (Chang et al. 2002). So, phylogenetic analysis based on the representative NRPS genes in Fig. 3 shows a high diversity of marine NRPS genes.

Antimicrobial Bioassay of Sponge-Associated Bacteria with NRPS Genes

According to Table 4, the antimicrobial tests against six pathogenic microbial indicators show that all of the 15 isolates with A domain genes have antimicrobial activities; in particular, most of the bacteria are active against multiple indicators with broad-spectrum antimicrobial activity. For instance, *Alcaligenes* sp. A11 is active against four indicators *P. fluorescens*, *S. aureus*, *E. coli*, and *B. subtilis* synchronously. The different antimicrobial spectra indicate the potential chemical diversity of biologically active metabolites of these sponge-associated bacteria and confirm the finding of diverse NRPS genes in these bacteria to some extent.

Generally, sponges are continuously exposed to harmful situations, which make them to develop a variety of chemical and immunological defense systems to protect themselves. Sponge-associated bacteria have been suggested to play an important role in the host's chemical defense by releasing biologically active metabolites (Müller et al. 2004). In this study, the revealed antimicrobial activities and NRPS genes suggest the possible role of bacterial symbionts in the host's antimicrobial chemical defense to some extent.

The antimicrobial activities of the 15 bacteria with NRPS genes are detected in this study; some NRPS gene relatives such as *B. pumilus* SAFR-032 (YP001485577), *B. subtilis* ATCC6633 (AAF08797), and *B. licheniformis* ATCC 10716 (AAD04757) are involved in the biosynthesis of peptides, and some bacterial relatives are able to synthesize peptides (Duitman et al. 1999; Konz et al. 1999; Rey et al. 2004). However, the role of these NRPS genes in the chemical defense mechanism for the sponge hosts against microorganisms cannot be revealed because of the lack of direct proofs. Even so, compared with the traditional method based on antimicrobial bioassay only, the combined molecular technique and bioassay strategy will be useful to obtain sponge-associated bacteria with the potential to synthesize bioactive compounds.

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