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Diversity of culturable actinobacteria isolated from marine sponge *Haliclona* sp.

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Abstract This study describes actinobacteria isolated from the marine sponge *Haliclona* sp. collected in shallow water of the South China Sea. A total of 54 actinobacteria were isolated using media selective for actinobacteria. Species diversity and natural product diversity of isolates from marine sponge *Haliclona* sp. were analysed. Twenty-four isolates were selected on the basis of their morphology on different media and assigned to the phylum Actinobacteria by a combination of 16S rRNA gene based restriction enzymes digestion and 16S rRNA gene sequence analysis. The 16S rRNA genes of 24 isolates were digested by restriction enzymes *Taq*I and *Msp*I and assigned to different groups according to their restriction enzyme pattern. The phylogenetic analysis based on 16S rRNA gene sequencing showed that the isolates belonged to the genera *Streptomyces*, *Nocardiopsis*, *Micromonospora* and *Verrucosispora*; one other isolate was recovered that does not belong to known genera based on its unique 16S rRNA gene sequence. To our knowledge, this is the first report of a bacterium classified as *Verrucosispora* sp. that has been isolated from a marine sponge. The majority of the strains tested belong to the genus *Streptomyces* and three isolates may be new species. All of the 24 isolates were screened for genes encoding polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS). PKS and NRPS sequences were detected in more than half of the isolates and the different ''PKS-I—PKS-II—NRPS'' combinations in different isolates belonging to the same species are indicators of their potential natural product diversity and divergent genetic evolution.

Keywords Marine sponge · Actinobacteria · 16S rRNA · RFLP · PKS · NRPS

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Introduction

Actinobacteria are widely distributed in terrestrial environments and have long been a source of commercially useful enzymes and therapeutically useful bioactive molecules (Cook and Meyers

2003), producing over half of the bioactive compounds in the Antibiotic Literature Database (Lazzarini et al. 2000).

The ocean covers approximately 70% of the surface of our planet and represents 95% of the biosphere (Bernan et al. 1997). Actinomycetes were once considered rare in the world's oceans, but have been found very widely distributed in recent years (Bull et al. 2005). Given that actinomycetes living in the ocean experience a dramatically different set of environmental challenges compared to their terrestrial relatives, it is not surprising that speciation has occurred and unique marine taxa are now being recognized (Jensen et al. 2005). There is now considerable evidence for the presence of a diverse assemblage of actinomycetes in the marine environment (Jensen et al. 1991; Takizawa et al. 1993; Moran et al. 1995; Colquhoun et al. 1998). It is likely that these unique taxa will produce the same or similar range of bioactive compounds as they share an evolutionary history with known producers (McVeigh et al. 1996; Ward and Goodfellow. 2004). Recent screening efforts focused on marine actinobacteria have revealed many new chemical entities and bioactive metabolites (Blunt et al. 2004; Salomon et al. 2004; Fiedler et al. 2005; Jensen et al. 2005) with a discovery rate that surpasses that of terrestrial actinobacteria (Bull et al. 2005). Marine actinomycetes, in particular, present a major resource for biotechnological search and discovery (Bull 2004; Fiedler et al. 2005; Jensen et al. 2005).

Sponges harbor large amounts of bacteria in their tissues that can amount to 40% of their biomass (Vacelet 1975; Vacelet and Donadey 1977), exceeding that of seawater by two to three orders of magnitude (Friedrich et al. 2001). Marine sponges produce a wide array of natural products and bioactive secondary metabolites (Conte et al. 1994; Perry et al. 1994; Brantley et al. 1995; Hirota et al. 1996; Faulkner 2000). As sessile filter-feeding animals, sponges are the largest sources of marine bioactive metabolites, accounting for up to 40% of all known natural marine products (Lee et al. 2001), a few of which are already at various stages in clinical trials for drug development (Haefner 2003). In some instances, these compounds may in fact be of microbial origin (Haygood et al. 1999; Moore 1999**)**. For example, *Vibrio* spp. associated with the sponge *Dysidea* sp. were shown to synthesize cytotoxic and antibacterial tetrabromodiphenyl ethers (Elyakov et al. 1991). The diketopiperazines associated with the sponge *Tedania ignis* were found to be produced by a *Micrococcus* sp. (Stierle et al. 1988). Recently, the antifungal peptide theopalauamide, isolated from the marine sponge *Theonella swinhoei*, was shown to be contained in a novel δ-proteobacterial symbiont of the sponge (Schmidt et al. 2000).

Recent studies using both culture-independent molecular approaches and culture-based methods demonstrated that novel, abundant actinobacteria assemblages are associated with the sponges *Rhopaloeides odorabile* (Webster et al. 2001), *Halichondria panacea* (Imhoff and Stöhr 2003) and *Hymeniacidon perleve* (Zhang et al. 2006). Unique community of actinomycetes have been isolated from marine sponges (Imamura et al. 1993; Bultel-Poncé et al. 1997). However, the investigation of sponge-associated actinobacteria is presently limited to a few sponges out of the over 15,000 marine species, which is insufficient to provide a general understanding of these microbes regarding their diversity, distribution, and ecology, as well as for further exploitation of this novel source of actinobacteria (Zhang et al. 2006). Thus sponges may provide a prolific source of novel actinomycetes for natural product screening. In addition, it is also conceivable that convergent evolution played a role in shaping the microbial community within sponges (Hentschel et al. 2002).

Sponge populations in the South China Sea are very diverse due to its tropical and subtropical climate. So far, only limited research attention has been paid to these populations. *Haliclona* sp. are widely distributed along the coast of the South China Sea near Hainan, China. To better understand antibacterial diversity associated with marine sponges and their microbial flora, we isolated actinobacteria from the inter-tidal marine sponge *Haliclona* sp. collected in the shallow water of the South China Sea. Actinobacteria were cultivated using a variety of media and their phylogenetic diversity was assessed using 16S rRNA gene sequencing and RFLP analysis.

Microbial and chemical diversity may, to an unknown extent, be uncoupled due to lateral gene transfer and deficiencies in current procedures for eliciting gene expression (Bull et al. 2005). Consequently, the presence of polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS) genes were screened using degenerate primers to study their associated potential capacity to synthesize diverse bioactive natural products.

Materials and methods

Sample site and sample collection

Sponge samples (1–2 kg) were collected from South China Sea (18° 13′ N; 109° 29′ E), and identified by Dr. K. J. Lee (Department of Biology, Hannam University, 133 Ojungdong, Daedukgu, Daejeon, Korea). The samples were kept in fresh seawater on ice and then stored at –20°C until analysis.

Selective Isolation

Sponge samples were ground and the supernatant fraction was diluted in series to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. It is noted that this strategy will recover both symbiont bacteria and those simply adherent i.e., living on the outside of the sponge cells. Samples (100 µl) from the various preparations were spread over the surface of Gause′s Medium No.1 (GM1), Glycerol Arginine Agar (GAA), Starch Casein KNO_3 Agar (SCKA), Streptomycete Medium (SM) and 2216E, as described in the Hand Book of Microbiological Media (Atlas and Park 2000), with the addition of 80% seawater. Isolation plates were incubated at 28°C for 3 weeks.

Maintenance, culture conditions and morphological grouping

Bacterial colonies bearing typical *Streptomyces*/actinobacterial morphology (colourful substrate mycelia, aerial mycelia, spores mass and pigment production) were selected and inoculated onto freshly prepared agar media and the inoculated plates were incubated for 2–4 weeks at 28°C. Isolates were maintained on the plates for short-term storage and as a suspension in 20% (v/v) glycerol at –20°C for long-term maintenance. All isolates were inoculated onto Gause's medium No.1 prepared in 80% seawater and fresh water, respectively, to observe the influence of seawater on actinobacterial growth. Purified isolates were then assigned to artificial groups based on aerial spore mass color, reverse pigment color and the color of any diffusible pigments.

DNA extraction

The total genomic DNA was extracted from all the isolates as described in Li and De Boer (1995).

Oligonucleotides and PCR amplification

All of the oligonucleotide primers (Table 1) were synthesized by SBS Genetech (China). The polymerase chain reaction was carried out on PTC200 (Bio-RAD) in a 20 µl volume. PCR mixtures included Taq Premix (TaKaRa Biotechnology (Dalian) Co., Ltd.) 10 µl, 1 µl F (10 µM), 1 µl R (10 µM), and 5% DMSO. After denaturation at 95°C for 1 min, amplification was performed with 30 cycles of 35 s at 94°C, 40 s at 55°C, 2 min at 72°C for 16S rRNA genes and PKS-I and 1 min at 72°C for NRPS and PKS-II, followed by a final extension at 72°C for 8 min.

16S rRNA restriction fragment length polymorphism analysis

Amplicons were digested with restriction enzymes *Taq*I and *Msp*I (TaKaRa) using procedures as follows: 3 µl PCR product was digested at 65°C for *Taq*I and 37°C for *Msp*I for 3 h in a PCR tube containing 1 µl 10 × Buffer, 1 µl 0.1% BSA (bovine serum A), and 5U restriction enzyme. DNA fragments were separated in 1.5% agarose (Biowest).

DNA sequencing and analysis

DNA sequencing was carried out by Invitrogen (China) and sequences were compared with those

Table 1 Oligonucleotide primers used in this study

Primer name	Sequences (5′–3′)	Target genes	Length of the target gene fragments (bp)	Reference
27F	1500R	5′-16S	rRNA	1,300–1,450
	GAGTTTGATCCTGGCTCAG-3′ 5′-AGAAAGGAGGTGATCCAGCC-3′			
Woese et al.	(1983)			
K1F M6R	5′-TSAAGTCSAACATCGGBCA-3′ 5′-CGCAGGTTSCSGTACCAGTA-3′	PKS-1	1,200–1,400	Ayuso-Sacido and Genilloud (2005)
IIPF6 IIPR6	5′-TSGCSTGCTTCGAYGCSATC-3′ 5′-TGGAANCCGCCGAABCCGCT-3′	PKS-2	600–700	Metsä-Ketelä (1999)
A3F A7R	5′-GCSTACSYSATSTACACSTCSGG-3′ 5′-SASGTCVCCSGTSCGGTAS-3′	NRPS	700–800	Ayuso-Sacido and Genilloud (2005)

in the GenBank database using the BLAST search program (http://www.ncbi.nlm.nih.gov/). Phylogenetic analysis was performed with program MEGA 3.1 (Molecular Evolutionary Genetics Analysis, Version 3.1) (Kumar et al. 2004). The tree topologies were evaluated by bootstrap analyses based on 1,000 replicates (Felsenstein 1985) and phylogenetic trees were inferred using the neighbor-joining method (Saitou and Nei 1987).

Results

Selective isolation

Gause's medium No.1, GAA, SCKA and SM were used to isolate actinobacteria from *Haliclona* sp. Actinobacteria were also found and isolated from *Haliclona* sp. using medium 2216E which was not designed for isolating actinobacteria. A total of 54 strains were isolated and 24 isolates were selected for further analysis on the basis of their colour group on the isolation medium.

16S rRNA gene RFLP analysis

PCR products, amplified from all 24 isolates using universal primers targeting the 16S rRNA gene, were digested using two restriction endonucleases (*Taq*I targeting the sequence CCGG and *Msp*I targeting TCGA) for analysis of their polymorphisms. The RFLP electrophoresis patterns of the 16S rRNA genes of the 24 strains revealed similar groups with both *Taq*I and *Msp*I. The 24 isolates digested with *Taq*I were assigned to six different RFLP patterns (Fig. 1 and Table 2). *Taq*I-based Group A is the dominant group harboring 15 isolates, while Groups B, C, D, E and F comprise 1–4 isolates, respectively. Six almost identical groups were detected when the 16S rRNA gene was digested using *Msp*I. Sixteen isolates were allocated to *Msp*I-based Group a, whereas *Msp*I-based Group d contained the same members as that of *Taq*I-based Group E, as did *Msp*I-based group e and *Taq*I-based Group F (Table 2). Isolate 1G103 showed a similar electrophoresis pattern to *Taq*I-based Group D, but grouped in *Msp*I-based Group a. The results suggested that a dominant culturable group may exist within sponge *Haliclona* sp., as symbionts or adherents (living on the outside of sponge cells), in the shallow water of the South China Sea.

Blast search and phylogenetic analysis based on 16S rRNA gene sequences

The 16S rRNA genes of the 24 isolates chosen as representatives of the diversity isolated were partially sequenced (Table 2; Genbank Accession numbers DQ994699-DQ994722). A BLAST analysis were carried out via blastn search through GenBank (http://www.ncbi.nlm.nih.gov) revealed that over 60% of the isolates (15 out of the 24) are members of the genus *Streptomyces*, which was the dominant actinobacterial genus within *Haliclona* sp. Seven isolates are either

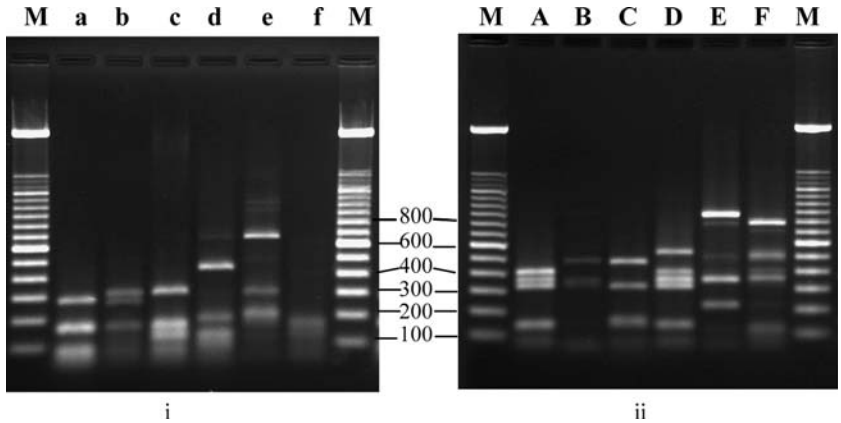

Fig. 1 The restriction fragment length polymorphism (RFLP) patterns of the 16S rRNA gene PCR products of actinobacteria isolated from the marine sponge *Haliclona* sp., M is 100 bp molecular weight marker (bp), The lane number corresponds to the RFLP pattern listed in Table 2. (**a**) Digested with the restriction enzyme *Msp*I, (**b**) Digested with the restriction enzyme *Taq*I

Table 2 Preliminary classification of culturable actinomycetes isolates from marine sponge *Haliclona sp.* Genbank Accession numbers of the 16S rRNA gene sequences for the 24 strains 1A1–1M83 are DQ994699 ~ **DQ994722**

Isolate codes	The most similar species (Accession number)	Identity (%)	RFLP group		PKS-I	PKS-II	NRPS	Selective medium
			*Taq*I	*Msp*I				
1A1*	*S. fradiae* (AB184069)	99	A	a	–	+	+	GAA
1C1*	*S. fradiae* (AB184069)	99	A	a	–	+	+	SCKA
1C2	*S. fradiae* (AB184069)	99	A	a	–	+	+	SCKA
1C3	*S. fradiae* (AB184069)	99	A	a	–	+	+	SCKA
1G1	*S. fradiae* (AB184069)	100	A	a	–	+	+	GM1
1M1*	*S. fradiae* (AB184069)	95	A	a	–	+	+	SM
1M9	*S. fradiae* (AB184069)	100	A	a	–	+	+	SM
1G101	*S. fradiae* (AB184069)	100	A	a	–	+	+	GM1
1C8*	*S. variabilis* (AB184884)	99	A	a	+	–	+	SCKA
1G102*	*Streptomyces* sp. (DQ663193)	99	A	a	+	+	+	GM1
1G103*	*Streptomyces* sp. (AF026081)	95	D	a	+	+	+	GM1
1A11*	*Actinomycetales* bacterium (DQ144217)	94	A	a	+	+	+	GAA
1E14	*S. griseoincarnatus* (AJ781321)	99	A	a	+	–	+	2216E
1E15*	*S. griseoincarnatus* (AJ781321)	98	A	a	+	–	+	2216E
1E33	*S. griseoincarnatus* (AJ781321)	98	A	a	+	–	+	2216E
1G4	*S. griseoincarnatus* (AJ781321)	99	A	a	+	+	+	GM1
1G61*	*Micromonospora carbonacea* (AY221498)	99	C	b	+	+	+	GM1
1G104*	*Micromonospora carbonacea* (X92606)	98	C	b	+	+	+	GM1
1G81	*Micromonospora carbonacea* (AY221498)	99	C	b	+	–	+	GM1
1G62*	*Micromonospora floridensis* (DQ126266)	100	C	c	+	+	+	GM1
1G67*	*Verrucosispora gifhornensis* (DQ416204)	99	B	f	–	–	–	GM1
1G68*	*Micromonospora* sp. (AY360169)	99	B	c	–	+	+	GM1
1G83*	*Nocardiopsis* sp. (AY764033)	99	E	d	+	+	+	GM1
1M83*	Uncultured bacterium (AF186414)	99	F	e	–	–	–	SM

* Strains used in phylogenetic tree constructed

"+" Represent PCR screening for target genes is positive and "–" is that of negative

members of *Micromonospora* (5 isolates), *Nocardiopsis* (1) or *Verrucosispora* (1), three major representative genera of actinobacteria (Table 2). However, 1M1 and 1A11 fail to assign to any known actinobacterial species, with the closest 16S rRNA gene sequence identity of 95% to

Streptomyces fradiae and 94% to *Actinomycetales* bacterium (DQ144217), respectively. The 1M83 16S rRNA gene sequence is homologous only to that of an uncultured actinobacteria harbored within sponge as symbionts, sharing up to 99% homology. The majority of the *Streptomyces* isolates (8 out of 15 isolates), representative of *Taq*I-based Group A, resemble the members of *S. fradiae* (Table 2), indicating that *S. fradiae*, or a closely related strain, is the dominant culturable microorganism, able to be detected by methods for actinobacterial isolation, in marine sponge *Haliclona* sp. collected from the coast of the South China Sea.

Further phylogenetic analysis was carried out on the 15 representatives with similarity to known actinobacteria aligned together with 23 representatives of authentic species or genera of actinobacteria (Fig. 2 and Tables 2, 3), and 1 unculturable marine microorganism. Figure 2 demonstrates the phylogenetic relationship among these isolates, together with the authentic species or type strains of actinobacteria as reference. All 15 isolates are grouped into five clusters at more than 10% dissimilarity value.

Cluster I resembled *Streptomyces* spp. comprising eight isolates and selected authentic species of *S. fradiae*^{T}, *Streptomyces variabilis*^{T}, *Streptomyces albus*^{T} and *Streptomyces griseoincarnatus*^{T} with a bootstrap value of 100%. IC8 can be comfortably assigned to *S. variabilis*, while our analyses suggest that an unclassified bacterium, the 16S rRNA sequence of which deposited at GenBank with a temporary name of Actinomycetales bacterium (DQ144217) should be also classified as *S. variabilis*. Isolates 1M1 and 1A11 are peripheral members of this clade. Both isolates may be new species as their homology with the closest reference strains is only between 94% and 95%. Their classification and characterization are undergoing further study.

Cluster II include 1G83 and *Nocardiopsis dassonvillei* with a bootstrap value of 100%. The genus *Nocardiopsis* has been shown to be phylogenetically coherent and to represent a distinct lineage within the radiation of the order *Actinomycetales* (Rainey et al. 1996)

Authentic species of cluster III and IV are members of Micromonosporaceae (Rheims et al. 1998), in particular, *Verrucosispora gifhornensis* (Cluster III), *Micromonospora carbonacea*, *Micromonospora floridensis* and *M. chalcea* (Cluster IV). One isolate (1G67) shared 99% homology with *V. gifhornensis*, while three other isolates shared 98–100% homology with *Micromonospora* spp.

One actinobacteria-like isolate, 1M83, forms a phylogenetically distinct lineage (Cluster V) with other culturable microorganisms of proteobacteria. This bacterium exhibits an actinobacteria-like colony, but phylogenetically, it has a great distance with a typical actinobacteria. Blast searching result indicates that 1M83 shared 99% 16S rRNA gene sequence homology with a non-culturable sponge symbiont. The other remotely related bacteria include uncultured bacteria phylogenetically related to Acidobacteriaceae, delta-proteobacteria and uncultivable Firmicutes. Therefore, reference type strains from these groups of microorganisms were chosen to construct the phylogenetic tree (Fig. 2). At present, the Gram stain morphology of 1M83 is not informative, and further work is being carried out for a precise description and taxonomy of this interesting bacterium, as well as its close relatives.

Detection, distribution and analysis of NRPS, PKS-I and PKS-II

Three sets of degenerate primers targeting genes encoding polyketide synthases (PKS-1 and PKS-2) and nonribosomal peptide synthetase (NRPS) were used to screen the biosynthetic potential of the 24 isolates, as identification of these genes provides indirect evidence of potential chemical diversity among these actinobacteria in terms of natural product drug discovery. Target sequences were amplified in more than half of the isolates (54% for PKS-I and 71% for PKS-II). NRPS sequences were detected in almost all of the isolates (92%). None of the target sequences were detected in isolates 1G67 and 1M83 (Table 2). The PCR amplicons and their origins were further confirmed by cloning and sequencing. An example of these data is given in Table 4.

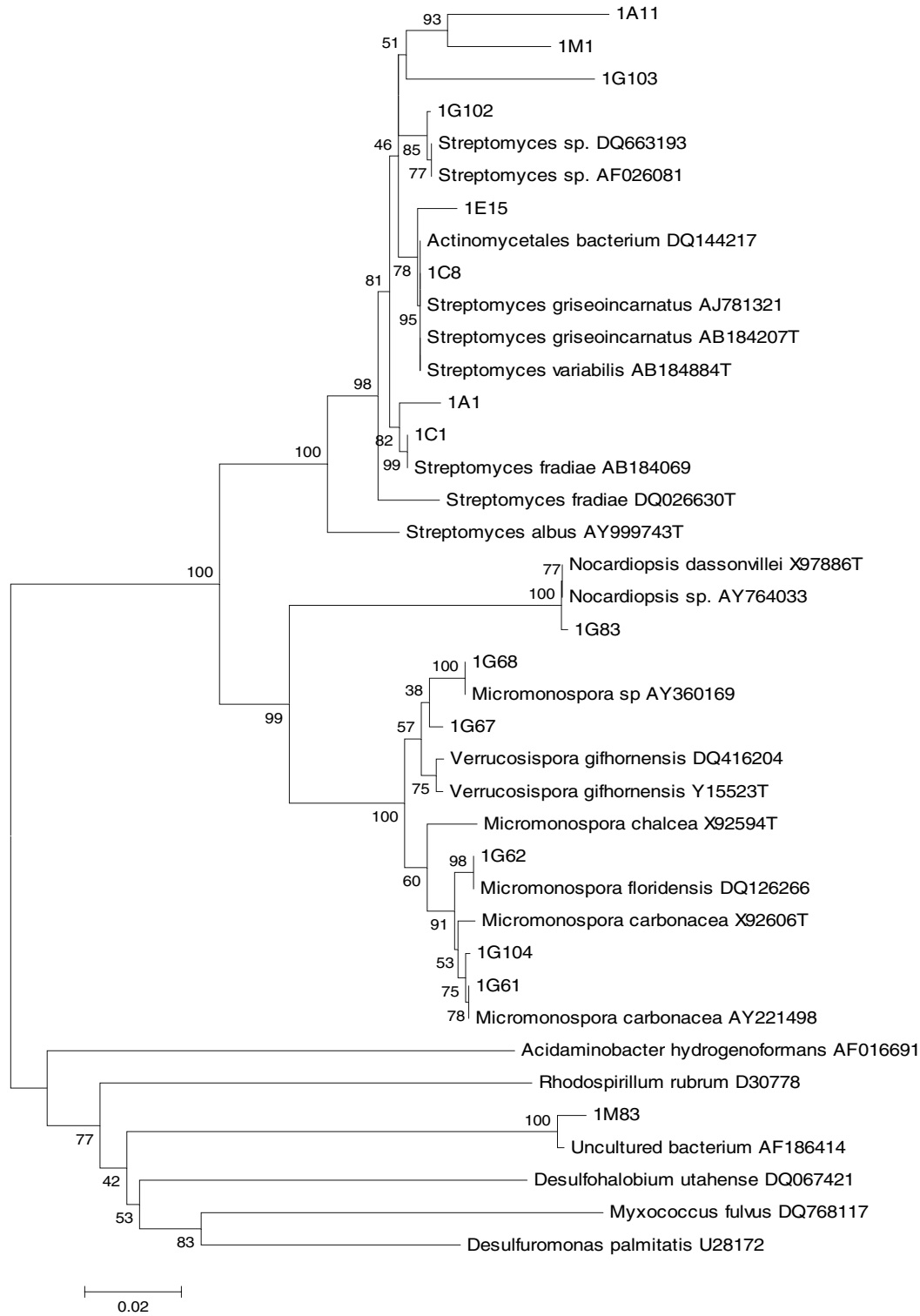

Fig. 2 Neighbor-joining phylogenetic representation of cultured actinomycetes and their closest NCBI (BLASTn) relatives based on the 16S rRNA gene sequences. Bootstrap values calculated from 1,000 resamplings using neighbor-joining are shown at the respective nodes when the calculated values were 50% or greater

Table 3 Type strains used in constructing the phylogenetic tree

ATCC; American Type Culture Collection, http://www.atcc.org

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen, http://www.dsmz.de/

NBRC: NITE Biological Resource Center, http://www2.nbrc.nite.go.jp

Type strain	Collection number	GenBank access number
Acidaminobacter hydrogenoformans	DSM 2784	AF016691
Desulfohalobium utahense	DSM 17720	DQ067421
Desulfuromonas palmitatis	ATCC 51701 = DSM 12931	U28172
Micromonospora carbonacea	DSM 43815	X92606
Micromonospora chalcea	ATCC 12452 = DSM 43026	X92594
Myxococcus fulvus	ATCC 25199 = DSM 16525	DQ768117
Nocardiopsis dassonvillei	ATCC 23218 = DSM 43111	X97886
Rhodospirillum rubrum	ATCC 11170	D30778
Streptomyces albus	ATCC 25426 = DSM 40313	AY999743
Streptomyces fradiae	ATCC10705	DQ026630
Streptomyces variabilis	NBRC 12825	AB184884
Verrucosispora gifhornensis	DSM 44337T	Y15523

Discussion

The majority of the isolates recovered in our study were representatives of the genus *Streptomyces*. Although the ''*Micromonospora-Rhodococcus-Streptomyces*'' group seems ubiquitous in cultured actinobacteria from marine environments (Maldonado et al. 2005), only *Micromonospora* spp. and *Streptomyces* spp. were isolated from the sponge *Haliclona* sp. Actinobacteria classified as *Micromonospora* spp. have been isolated previously from a marine sponge by Zhang et al. (2006).

To our knowledge, this is the first time that a strain of the rarely recovered genus *Verrucosispora* has been isolated and cultured from a marine sponge. The 16S rRNA gene sequence of isolate 1G67 shares 99% homology with that of *Verrucosispora gifhornensis* (Table 2), and is thus phylogenetically closely related to this species (Fig. 2). The genus *Verrucosispora* belongs to the suborder *Micromonosporineae*. The first representative of this genus isolated from terrestrial samples was described in 1998 (Rheims et al. 1998) and a second member of this new taxon was isolated from sediment sample collected from the Sea of Japan at a depth of 289 m (Riedlinger et al. 2004). The strains isolated from sea sediment seem to be a promising new taxon from which highly bioactive metabolites can be expected, in contrast to the terrestrial strains (Fiedler et al. 2005). The increasing numbers of rare actinobacteria found from marine sponges indicate that sponges are potentially unique sources of novel actinobacteria (Zhang et al. 2006) with promising potential to produce highly bioactive metabolites.

16S rRNA gene RFLP analysis can effectively reduce the number of isolates needing to be sequenced during screening for diversity. In the current study, we used two restriction endonucleases *Taq*I and *Msp*I that specifically recognize the sequence ''CCGG'' and ''TCGA''. The results of the different RFLP patterns obtained allowed us to effectively differentiate the strains into distinct groups of actinobacteria. This rapid and convenient method can be very useful in grouping actinobacterial isolates efficiently. Although caution must be taken when using the RFLP approach for a complete phylogenetic analysis (Zhang et al. 2006) and some strains with identical RFLP pattern displayed distinguishable 16S

Table 4 PKS/NRPS sequences derived from isolate 1G62

PKS/NRPS Codes	Genbank accession numbers	The most similar species (Accession number)
62I-4P	EF451568	*Streptomyces* sp. polyketide synthase gene (AF082100)
62II-40	EF451569	*Saccharopolyspora hirsuta* polyketide synthase gene (M98258)
62S-26	EF451570	*Streptomyces coelicolo*r A3(2) putative peptide synthetase gene (AL939105)

rRNA sequences (Table 2). Nevertheless the results of sequencing are consistent with the results of the 16S rRNA-based RFLP analysis. Actinobacterial isolates could be grouped at the genus level using restriction endonucleases *Taq*I and *Msp*I and some of these groupings held up at the subgenus level (Table 2 and Fig. 2). Of the five strains belonging to the genus *Micromonospora*, two different isolates 1G62 and 1G68 were distinguished from the three *Micromonospora* spp. strains in the study. Rare actinobacterial isolates are easy partitioned at the genus level, such as strains 1G83, 1G67 and 1M83, consistent with previous reports (Cook and Meyers 2003; Zhang et al. 2006). The results also showed that the use of different kinds of restriction enzyme could be helpful to fine tune assignment of sponge isolates at the genus and subgenus level (Lanoot et al. 2005). Isolates belonging to the genus *Streptomyces* were difficult to partition at the subgeneric level, though 1G103 was distinguished from the other 14 strains belonging to the genus *Streptomyces* in this study. 16S rRNA gene-based RPLP analysis has been reported to not distinguish strains belonging to *Streptomyces* at the subgeneric level (Cook and Meyers 2003; Lanoot et al. 2005; Zhang et al. 2006).

Strain 1M83 may be a new taxon with an actinobacteria-like colony. So far, no closely related culturable bacteria have been found based on 16S rRNA BLAST searches in GenBank. A previous study has revealed that homologous sequences of unculturable bacteria do exist in different sponge species (Hentschel et al. 2002) and intertidal sediments (Musat et al. 2006) detected using culture-independent methods. A detailed study is being carried out for characterization and classification of a group of isolates belonging to this new taxon.

The strategy of prescreening for PKS and NRPS can be used to assist in the discovery of bacterial natural product diversity (Pathom-aree et al. 2006) and often the natural product diversity reflects bacterial genetic diversity. Fifteen *Streptomyces* isolates in this study fell into different phylogenetic groups, some of them assigned to the same species and sharing identical RFLP pattern, but different ''PKS-I—PKS-II—NRPS'' combinations were shown after screening by PCR amplification, such as isolates assigned to *S. griseoincarnatus* and *Micromonospora carbonacea*. Thus the results suggest that their natural product diversity and genetic diversity has diverged.

PKS and NRPS were not detected from strains 1G67 and 1M83 using the selected degenerate primer pairs. These primer pairs have been used in a broad survey of these genes in a similar study (Ayuso-Sacido and Genilloud 2005). The absence of amplicons might indicate that some of the isolates lack NRPS, PKS-I and PKS-II genes, though it is also possible that these specific degenerate primer pairs might not be suitable to amplify these genes. Furthermore, not all NRPS genes are involved in the biosynthesis of bioactive secondary metabolites; indeed, the products of such genes may be involved in functions such as iron metabolism or quorum sensing (Finking and Marahiel 2004). It is also possible that the genes detected by PCR are nonfunctional. Nevertheless, the strategy of prescreening with PCR primers, which target genes potentially encoding for the biosynthesis of bioactive compounds, is an effective approach for detecting novel and useful secondary metabolites (Ketela et al. 1999, 2002; Courtois et al. 2003; Liu et al. 2003; Ginolhac et al. 2004; Pathom-aree et al. 2006).

All of the isolates recovered in this study can grow on media prepared with both seawater and freshwater though some of them grew slower and with altered morphology in media without seawater. Previous studies also found that most culturable actinobacteria isolated from the sea do not show an absolute requirement for seawater to grow (Mincer et al. 2002; Maldonado et al. 2005; Zhang et al. 2006). For some of the strains reported in this paper we could not exclude their terrestrial origin absolutely, though some of the actinobacterial clades are definitely indigenous and not washed in from terrestrial sources (Han et al. 2003; Warnecke et al. 2004).

In conclusion, these results provide further evidence that marine sponges are a rich and novel source for actinobacteria and, potentially, natural products. The culturable actinobacteria, isolated from the marine sponge *Haliclona* sp. in the South China Sea belong to four Actinobacteria genera, and one isolate can not assign to any

known culturable bacterial group. *Streptomyces* appears to be the dominant genus among symbionts and adherents present in *Haliclona* sp. in the South China Sea. Prescreening for PKS and NRPS revealed extensive metabolic potential in this group of diverse actinobacteria. The isolation of culturable actinobacteria from marine sponges can contribute to our knowledge of sponge-associated actinobacteria and further increase the pool of actinobacteria available for bioactive natural products screening.

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