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ORIGINAL ARTICLE

Marine sponge *Craniella austrialiensis*-associated bacterial diversity revelation based on 16S rDNA library and biologically active Actinomycetes screening, phylogenetic analysis

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Keywords
Actinomycetes, antimicrobial potential, *Craniella austrialiensis*, phylogenetic analysis, RFLP, 16S rDNA library.

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Abstract

Aims: The aim of this study was to investigate the bacterial diversity associated with the sponge *Craniella australiensis* using a molecular strategy and isolating Actinomycetes with antimicrobial potentials.

Methods and Results: The bacterial diversity associated with South China Sea sponge *C. austrialiensis* was assessed using a 16S rDNA clone library alongside restriction fragment length polymorphism and phylogenetic analysis. It was found that the *C. austrialiensis*-associated bacterial community consisted of alpha, beta and gamma-*Proteobacteria*, *Firmicutes*, *Bacteroidetes* as well as *Actinobacterium*. Actinomycetes were isolated successfully using seawater medium with sponge extracts. According to the BLAST and phylogenetic analysis based on about 600-bp 16S rDNA sequences, 11 of the representative 23 isolates closely matched the *Streptomyces* sp. while the remaining 12 matched the Actinomycetales. Twenty Actinomycetes have antimicrobial potentials, of which 15 are found to possess broad-spectrum antimicrobial potentials.

Conclusions: The sponge *C. austrialiensis*-associated bacterial community is very abundant including *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacterium* while Actinomycetes is not predominant. Artificial seawater medium with sponge extracts is suitable for Actinomycetes isolation. Most of the isolated *C. austrialiensis*-associated Actinomycetes have a broad spectrum of antimicrobial activity.

Significance and Impact of the Study: This study revealed the diversity of the bacterial community and the isolated Actinomycetes with antimicrobial potentials associated with sponge *C. australiensis*.

Introduction

It is well known that Actinomycetes boast an unparalleled ability to produce diverse secondary metabolites and thus are best known as a source of antibiotics. By the 1980s, almost 70% of the world's naturally occurring antibiotics were attributed to Actinomycetes (Okami and Hotta 1988). Today, nearly all the known Actinomycetes come from terrestrial soils and contribute significantly to the turnover of complex biopolymers, such as hemicellulose, pectin, keratin, and chitin besides the importance in soil ecology. In the last two decades, the rate of discovery of new lead compounds from existing genera obtained from terrestrial sources has decreased while the number of drug-resistant pathogens increased dramatically. At present, the finding of rare or novel marine Actinomycetes has become a major focus in the search for the next generation of pharmaceutical agents (Bull *et al.* 2000).

Marine sponge is known as an important source for marine drug development (Sipkema *et al.* 2005).

Accumulated evidence suggests that micro-organisms could well be the true source of at least some of the biologically active metabolites (Imhoff and Stohr 2003). Hence, sponge-associated micro-organisms are an important source for finding marine potential active compounds and biological function (Osinga *et al.* 2001).

It is widely accepted that culture-based technique is inadequate for the studying of bacterial community diversity because most of the bacteria are very difficult to culture by current and traditional techniques. The recent surge of research in molecular microbial ecology provides compelling descriptions on complete microbial community composition and can indicate possible nutritional requirements and physiological niches of many micro-organisms according to the information already available for known phylogenetic relatives (Distel *et al.* 1991). 16S rRNA gene-based approaches have enormously enhanced our knowledge about the diversity of environmental microbial communities, such as marine sponge-associated microbial diversity (Webster *et al.* 2001a,b; Hentschel *et al.* 2002). This may also be helpful for the experimental manipulation of culture conditions to provide the correct growth conditions for targeted bacteria (Hentschel *et al.* 2003).

To date, studies on sponge-associated Actinomycetes are rare because of their low abundance in sponges and the difficulty of cultivation *in vitro*. Sponge *Craniella* sp. was first reported by Marsden (1975); the relative study was very rare and only involved sponge-derived compounds (Mokashe *et al.* 1994). In the case of sponge *Craniella australiensis*, no report has been found to date except for a study on its predominant bacterial community using a denaturing gradient gel electrophoresis (DGGE) strategy (Li *et al.* 2006). Hence, the aim of this study was to investigate the diversity of the bacterial community associated with marine sponge *C. australiensis* using a molecular strategy based on 16S rRNA gene library and by the isolation of Actinomycetes with antimicrobial potentials.

Materials and methods

Sponge sample

Marine sponge *C. australiensis* (Porifera, Class Demospongiae, Order Choristida, Family Craniellidae) was collected by scuba diving at a depth of about 20 m in the South China Sea around Sanya Island and enclosed in axenic bags immediately. Before DNA extraction and bacterial isolation, sponge samples were stored at 4°C. The collected sponge was identified by Prof Jin-He Li in the Institute of Oceanology, Chinese Academy of Sciences.

DNA extraction and 16S rDNA clone library construction

Total DNA was extracted from lyophilized sponge tissue using the protocol described by Schmeisser *et al.* (2003). Amplification of 16S rDNA was performed in Master Cycler Gradient (HYBAID, Middlesex, UK) with the eubacterial primers 27f (5′-GAGAGTTTGATCCTGGCT CAG-3′) and 1492r (5′-CTACGGCTACCTTGTTACGA-3′). Cycling conditions were as follows: initial denaturation at 94°C for 3 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 *c.* 1500 bp were purified by electrophoresis in a 1% (wt/vol) agarose gel and recovered using a gel purification kit (Shenergy Biocolor Bioscience & Technology Company, Shanghai, China).

The PCR products were purified with a PCR purification mini kit (Shenergy Biocolor Bioscience & Technology Company, China) and were ligated into the pUCmT vector, and transformed into $CaCl_2$-competent *Escherichia coli* DH5α. The positive recombinants were screened on X-Gal (5-bromo-4-chloro-3-indoly-β-D-galactopyranoside)-IPTG (isopropyl-β-D-thiogalactopyranoside)-ampicillin-tetracycline indicator plates by colour-based recombinant selection. Positive clones were identified by PCR amplification with pUCmT vector primer pairs T7 (5′-TAATACGACTCACTATAGGG-3′) and M13 (5′-CAGGAAACAGCTATGACC-3′) using the same program as that for 16S rDNA amplification. Ninety-five recombinant clones that showed positive signals were used in the following analyses.

Restriction fragment length polymorphism analysis and 16S rDNA sequencing and phylogenetic analysis

PCR products were characterized by single digestion with the restriction endonucleases, *Csp6*I and *Hinf*I (MBI). Clones with similar restriction patterns were grouped together, and random clones from each group were chosen for sequencing: ABI3730 DNA Sequencer (USA) with primer 1492r from Bioasia Biotechnology Company, China. Partial sequences were compiled and aligned using the CLUSTALX program. Finally, sequences of *c.* 600 bp, suitable for phylogenetic analysis, were checked for chimera formation (CHECK_CHIMERA online analysis of Ribosomal Database Project II) and then compared with sequences in the available database by using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul *et al.* 1990) to determine approximate phylogenetic positions. The phylogenetic tree was generated using the neighbour-joining method with 1000 resampling bootstrap analyses using Mega II software.

Isolation and phylogenetic analysis of sponge-associated Actinomycetes

First, sponge extract solution was prepared as follows: 2 kg of the sponge *C. australiensis* was dipped in 10 000 ml of methanol for 1 month, 200 ml of the extraction solution was concentrated under reduced pressure to obtain 1·80 g of crude sponge extracts and then the extracts were dissolved in 30 ml of sterilized distilled water. Isolation medium consisted of 18 g of agar, 1000 ml of artificial sea water (ASW) containing NaCl (26·518 g), $MgCl_2$ (2·447 g), $MgSO_4$ (3·305 g), $CaCl_2$ (1·141 g), KCl (0·725 g), $NaHCO_3$ (0·202 g), NaBr (0·083 g), ddH_2O (1000 ml) and sponge extracts solution at a final concentration of 0·1% (v/v). All the plates were supplemented with nystatin (10 mg l^{-1}) and K_2CrO_7 (0·05 g l^{-1}) after autoclaving to inhibit bacterial and fungal growth. Whatman No. 1 sterile filter paper was placed on the agar, and the ground sponge pieces (1–3 mm^3) were dispersed on the surface of the paper. The plate was incubated in a humidified chamber at 28°C, which favours bacterial growth for weeks. Following incubation, colonies selected according to the morphological characteristics on plates were streaked repeatedly on Gause I medium (soluble starch 20 g, KNO_3 1 g, NaCl 0·5 g, K_2HPO_4 0·5 g, $MgSO_4$ 0·5 g, $FeSO_4$ 0·01 g, ddH_2O 1000 ml, agar 18 g) prepared with ASW to obtain pure isolation. After isolation, the isolates were cultured using M1 medium (Mincer *et al.* 2002), containing 10 g of starch, 4 g of yeast extract, 2 g of peptone, 18 g of agar, and 1 l of ASW.

Strains were preliminarily identified according to the traditional morphological criteria (Goodfellow and Cross 1984). Then the representative isolates were identified based on 16S rRNA gene sequencing. Genomic DNA of the isolated Actinomycetes was prepared using a method described by Magarvey *et al.* (2004). The processes of amplification of the 16S rRNA gene, purification, sequencing, and phylogenetic analysis were similar to those mentioned previously.

Antimicrobial potentials screening of sponge-associated Actinomycetes

Twenty-three pure actinomycetes isolates were selected according to the morphology and colour characteristics on plates for their antimicrobial potential investigation by agar

Table 1 Similarity to the closest relative in GenBank of bacterial 16S rDNA clone sequences retrieved from sponge *Craniella australiensis*

		Result of BLAST analysis		
Clone	Accession number	Length (bp)	Identity (%)	Closest relative and its accession number
Gamma-Proteobacteria				
C-Clone7	DQ180159	622	100	Uncultured Arctic sea ice bacterium (AY165598)
C-Clone8	DQ180161	622	100	*Halomonas* sp. HA-T (AB104435)
C-Clone18	DQ180151	621	99	*Enterobacter hormaechei* subsp. *steigerwaltii* (AJ853890)
C-Clone23	DQ180152	621	100	Uncultured soil bacterium (AF423294)
C-Clone79	DQ180160	620	99	*Pseudoalteromonas* sp. 01/121 (AJ874345)
C-Clone134	DQ180148	621	100	*Pseudoalteromonas* sp. ANT9388 (AY167333)
Beta-Proteobacteria				
C-Clone6	DQ180157	616	100	*Alcaligenes* sp. YcX-20 (AY628412)
C-Clone54	DQ180156	616	98	*Alcaligenes* sp. YcX-20 (AY628412)
C-Clone121	DQ180143	620	99	Uncultured beta-proteobacterium (AF534429)
Actinobacterium				
C-Clone129	DQ180146	622	95	Uncultured actinobacterium (DQ028394)
Firmicutes				
C-Clone13	DQ180147	620	98	*Carnobacterium funditum* (S86170)
C-Clone138	DQ180149	623	99	*Bacillus* sp. N-1 (AB043851)
Alpha-Proteobacteria				
C-Clone116	DQ180142	598	99	Marine bacterium SCRIPPS_101 (AF359537)
C-Clone123	DQ180144	620	99	*Hyphomicrobium* sp. P2 (AF148858)
C-Clone140	DQ180150	600	97	*Rhodobacter* sp. QSSC1–20 (AF170736)
Bacteroidets				
C-Clone3	DQ180153	616	100	*Bizionia paragorgiae* (AY651070)
C-Clone4	DQ180154	616	98	*Cytophaga* sp. MGP-8AN (AF530156)
C-Clone43	DQ180155	622	98	*Aequorivita ferruginea* (AY027804)

BLAST, Basic Local Alignment Search Tool.

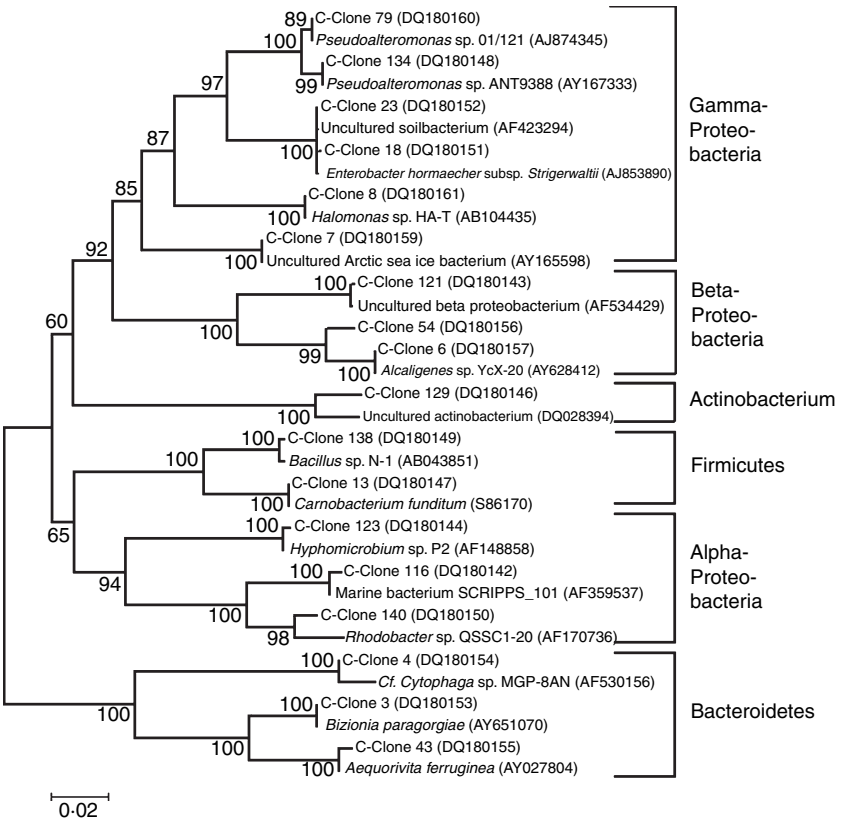

Figure 1 Neighbour-joining phylogenetic tree based on about 600 bp of 16S rRNA gene sequences from the clone library. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled data sets. Scale bar represents 0·02 substitutions per nucleotide position.

diffusion method against six indicator strains including gram-positive and gram-negative bacteria and fungi: *E. coli* (AS 1.3373), *Staphylococcus aureus* (AS 1.2465), *Pseudomonas fluorescens* (AS 1.55), *Bacillus subtilis* (AS 1.3343), *Candida albicans* (AS 2.2086), and *Pacecilomyces variotii* (AS 3.776), which were purchased from the Chinese Biodiversity Information Center in the Institute of Microbiology, Chinese Academy of Sciences, cultured on M1 medium plates (Mincer *et al.* 2002) using sterile distilled water instead of ASW. The indicator strain cultures were spread on plates and then the isolated *Actinobacterium* was incubated on them at 28°C for 3–4 weeks. The antimicrobial activity was evaluated by the formation of an inhibition zone on the plate around the *Actinobacterium*. All the tests were repeated three times with one single test in replicates. The zone diameter was an average of six results.

Results

Bacterial diversity based on 16S rRNA gene library

Amplification of the 16S rRNA genes from the sponge-derived total DNA yielded about 1500-bp fragments. Ninety-one clones were compared by RFLP analyses, resulting in 21 restriction patterns. On the whole, 18 representative clones were chosen for partial sequencing and submitted to the GenBank. The obtained accession numbers are listed in Table 1. The results based on about 600-bp 16S rDNA sequences indicated abundant bacterial diversity in sponge *C. australiensis* (Table 1). All of the sponge-derived 16S rDNA sequences showed higher than 95% to their nearest relatives in the GenBank.

According to Fig. 1, the majority of sponge-derived microbial sequences were related to *Bacteroidetes* division ($n = 39$; 43·9%) and gamma-*Proteobacteria* ($n = 31$; 34·0%). Clones affiliated with *Firmicutes* ($n = 8$; 8·8%), alpha-*Proteobacteria* ($n = 7$; 7·7%), beta-*Proteobacteria* ($n = 5$; 5·5%) were also observed. Sequences related to *Actinobacterium* were only a minor component of the gene library (Fig. 1).

Isolation and phylogenetic analysis of sponge-associated Actinomycetes

Due to the extremely low abundance of sponge-derived Actinomycetes showed by the bacterial diversity survey of *C. australiensis* (Fig. 1), an isolation approach simulating natural conditions was employed using medium containing only seawater and sponge extracts. After 3–4 weeks of incubation, lawns of spores were seen on the appearance

Table 2 Similarity to the closest relative in GenBank of 16S rDNA sequences of Antinomycetes associated with sponge *Craniella australiensis*

		Result of BLAST analysis		
Strain	Accession number	Length (bp)	Identity (%)	Closest relative and its accession number
DA01	DQ180118	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA02	DQ180119	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA03	DQ180120	599	98	*Actinomycetales bacterium* H07 (AY944257)
DA04	DQ180121	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA05	DQ180122	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA06	DQ180123	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA07	DQ180124	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA08	DQ180125	599	98	*Streptomyces* sp. VA26256_03 (AY436366)
DA09	DQ180126	599	98	*Streptomyces* sp. VA26256_03 (AY436366)
DA10	DQ180127	599	99	*Actinomycetales bacterium* H07 (AY944257)
DA11	DQ180128	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA12	DQ180129	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA13	DQ180130	599	99	*Actinomycetales bacterium* H07 (AY944257)
DA14	DQ180131	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA17	DQ180132	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA18	DQ180133	599	98	*Streptomyces* sp. FXJ23 (AY314785)
DA19	DQ180134	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA20	DQ180135	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA21	DQ180136	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA22	DQ180137	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA23	DQ180138	599	99	*Streptomyces* sp. FXJ23 (AY314785)
DA24	DQ180139	599	100	*Actinomycetales bacterium* H07 (AY944257)
DA25	DQ180140	599	98	*Streptomyces* sp. FXJ23 (AY314785)

BLAST, Basic Local Alignment Search Tool.

of the filter paper. After purifying twice on Gause I medium, a total of 23 representative Actinomycetes isolates were chosen for further identification and biological activity study according to the different morphologic and colour characteristics.

The BLAST analysis of 16S rRNA gene sequence (*c.* 600 bp) of the 23 strains (DQ180118–180140 in GenBank) with the known sequences in the GenBank database revealed homologies of greater than 98% to members of Actinomycetes (Table 2). Though some isolates, such as DA01–02, DA04–07, DA08–09, DA17–18, DA19–21, DA22, and DA24 match the same relative with the same similarity in Table 2, they were included in the phylogenetic tree in Fig. 2 as the sequenced 16S rDNA segments are about 600 bp and the morphological or biochemical characteristic profiles of these strains are different to some extent. Based on the phylogenetic analysis, 11 strains closely matched the *Streptomyces* sp., while *Streptomyces* sp. strain DA08 and *Streptomyces* sp. strain DA09 showed remote genetic distance from other strains.

Antimicrobial potential screening

The tests of 23 Actinomycetes strains against six pathogenic microbial indicators showed that 20 out of 23 Actinomycetes have antimicrobial potential especially against *P. fluorescens*. Particularly, 15 strains were found to be active against multiple indicator strains with broad-spectrum antimicrobial activities, for example, *Streptomyces* sp. DA21 and DA22 exhibited evident inhibition zone against all six indicators synchronously; strains DA01, DA02, DA09, DA11, DA13, DA14, DA17, DA18, DA19, DA23, DA24, and DA25 exhibit against four indicators.

Discussion

The bacterial community structure obtained conforms to our previous study by DGGE fingerprint approach (Li *et al.* 2006) and is also similar to the results of Webster *et al.* (2001a,b) and Hentschel *et al.* (2003) with *Proteobacteria* as the predominant component. The comprehensive 16S rRNA-based molecular approach is valuable in revealing the sponge-associated bacterial diversity. The constructed 16S rDNA library in this study contained sequences of specific phylogenetic clusters of *Pseudoalteromonas*, *Bacillus*, and *Actinobacteria* that had previously been isolated form marine sponge (Chelossi *et al.* 2004). Marine Actinomycetes were also found in marine sponges by Webster *et al.* (2001a) and Montalvo *et al.* (2005). Particularly, the correlative nearest relatives to C-clone 7,

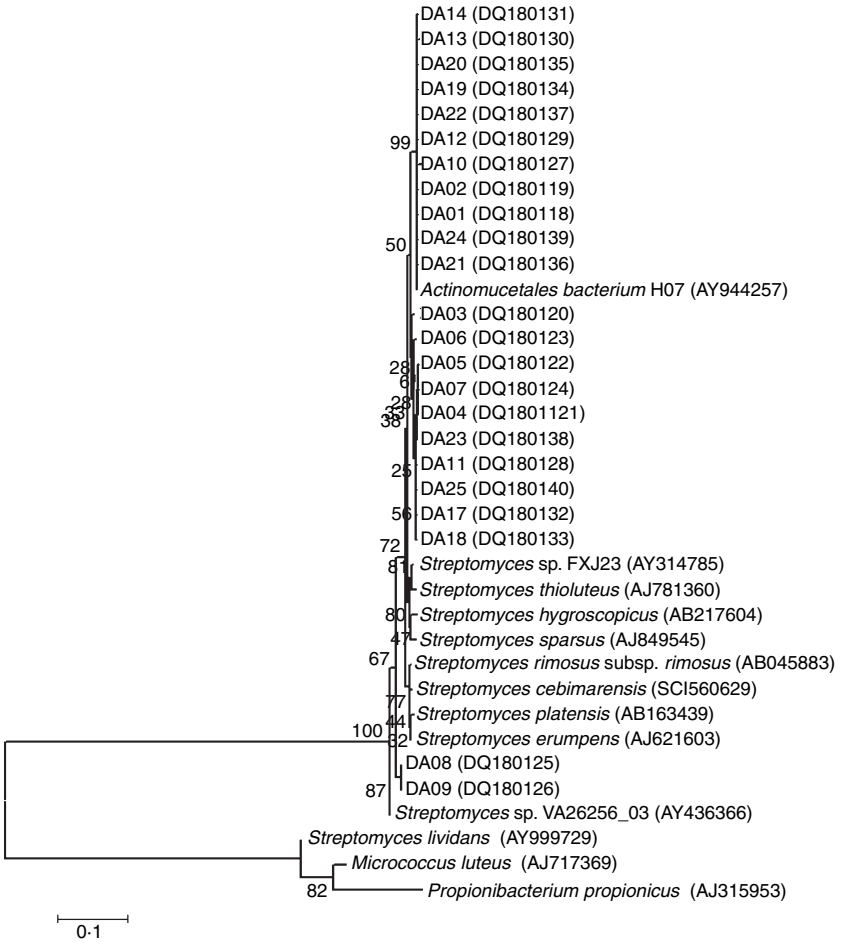

Figure 2 Neighbour-joining phylogenetic tree based on about 600 bp of 16S rRNA gene sequences of 23 Actinomycetes isolates from marine sponge *Craniella australiensis*. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled data sets. Scale bar represents 0·1 substitutions per nucleotide position. *Propionibacterium propionicus*, *Streptomyces lividans*, and *Micrococcus luteus* were used as out groups.

C-clone 23, C-clone 121, and C-clone 129 are uncultured bacteria and C-clone 129 may be an unknown bacterium with the lowest similarity being 95% to the relative indicating a big challenge in the recovery of sponge-associated bacteria.

Actinomycetes are widely distributed in the marine environment displaying competitive biosynthetic capabilities (Zheng *et al.* 2000). However, a majority of marine Actinomycetes are very difficult to isolate because of their low abundance and special nutrition requirements. In this study, a novel isolating procedure imitating the natural nutrition state of Actinomycetes within the sponge host resulted in the successful isolation of Actinomycetes from the sponge *C. australiensis*.

Generally, sponges are continuously exposed to a wide diversity of potentially harmful situations, which make them develop a variety of chemical and immunological defense systems to protect themselves. The roles played by sponge-associated micro-organisms in the host chemical defence by low molecular metabolites or toxins and enzyme attack have been suggested by Müller (2003), which was also strongly suggested by the stronger, multiple antimicrobial potentials of sponge *C. australiensis*-associated Actinomycetes in this study. Hence, sponge *C. australiensis*-associated Actinomycetes should be a promising resource for marine drug development.

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