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Research Article

RESEARCH ARTICLE

Diversity and antimicrobial potential of *Actinobacteria* isolated from diverse marine sponges along the Beibu Gulf of the South China Sea

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One sentence summary: A total of 363 bacterial isolates, including 123 actinobacterial strains, were isolated from 49 marine sponges from Beibu Gulf, South China Sea, and their antimicrobial potential and functional gene resources were analyzed.

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ABSTRACT

Marine sponge-associated microorganisms have proven to be a very promising source of biologically active and pharmaceutically important natural products. In this study, we investigated the diversity and antibacterial potential of bacteria from 49 sponge species isolated from the Beibu Gulf, South China Sea, belonging to 16 genera and several unidentified taxa. Using a variety of selective media, 363 strains with different morphologies were identified to six bacterial taxa, including Proteobacteria (α-subgroup 85 and γ-subgroup 59), Actinobacteria (123), Firmicutes (90), Bacteroidetes (5) and Brevundimonas (1). Media ISP2 and R2A were the most effective for isolating Actinobacteria. One hundred and twenty-three actinobacterial strains clustered into 21 genera identified by 16S rDNA gene sequencing, most of which were from the genus *Microbacterium*, followed by *Pseudonocardia*, *Streptomyces*, *Kocuria*, *Aeromicrobium*, *Brachybacterium* and *Nocardiopsis*, constituted 82% of total actinobacterial isolates. By using the minimal medium, 92 actinobacterial isolates showed antimicrobial activities, and 51 strains displayed moderate to strong antimicrobial activity that inhibited the growth of more than half of the bacteria tested in this study. Functional genes related to secondary metabolites were screened, revealing that 10% (12/123) of actinobacterial isolates contained PKS-KS genes, 18% (22/123) harbored NRPS-A genes and 6% (7/123) had hybrid PKS-NRPS gene clusters. The sponges *Haliclona* sp., *Callyspongia* sp. and *Desmacella* sp., belonging to class Demonspongiae, and *Leucaltis* sp. from the class Calcarea, were dominant hosts, harboring the most diverse actinobacterial genera with stronger antimicrobial activities and more diverse PKS/NRPS genes.

Keywords: antibacterial activity; associated actinobacteria; diversity; functional genes; marine sponges

INTRODUCTION

Marine sponges, the most primitive multicellular metazoan animals, are sessile organisms that efficiently filter-feed organisms from the surrounding water (Kiran et al. 2018). Sponges have been studied to exploit their potential chemical diversity for the development of new medicines, with more than 200 new sponge-derived metabolites reported each year (Taylor et al. 2007). As natural microbial fermenters, sponges harbor a large

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community of diverse microorganisms that represent up to 50–60% of the sponge biomass (Thomas, Kavlekar and LokaBharathi 2010). These bacteria represent most microbial phyla, including 41 bacterial and three archaea phyla, as well as fungi and microalgae (Thomas et al. 2016). Secondary metabolites have been increasingly shown to be produced by sponge-associated microorganisms rather than by the sponges themselves, including polyketides, peptides and alkaloids, numerous examples of which possess attractive antitumor, antimicrobial, antifungal, anti-infective properties (Graça et al. 2015). To date, more than 5300 different natural compounds have been discovered from sponges and sponge-derived microorganisms, especially sponge-derived actinobacteria due to their unusual biological activity to treat various diseases and being the major source of antibiotics (Bibi et al. 2017). These compounds may be used as part of defensive strategies to escape predators, playing a crucial role in sponge survival and promoting their dominant position in the marine ecosystem (Garate et al. 2015, Scherlach and Hertweck 2018).

Over the past few decades, marine actinobacteria have been shown to be one of the most important secondary metabolite-producing microbial groups associated with sponges and are therefore important to the pharmaceutical industry (Xi et al. 2012). Although fewer actinobacterial isolates exhibit clinically relevant activities than fungi, many of the compounds produced by actinobacteria have yet to be characterized (Thomas, Kavlekar and LokaBharathi 2010). Once characterized, the distribution of clinically active compounds obtained from actinobacteria and fungi may change. Polyketides and nonribosomal peptides are well-studied examples of biological metabolites obtained from marine sponge-derived microbes (He et al.). These two classes of compounds are potentially related to polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) genes (Amoutzias, Chaliotis and Mossialos 2016), which encode large, multi-module and multidomain enzymes. In fact, the identification of PKS and NRPS genes from sponge-associated actinobacteria has provided compelling evidence that a diverse array of bioactive metabolites are synthesized by PKS and NRPS gene clusters (Zhou et al. 2011). Therefore, the search for the genes responsible for the expression of these enzymes is indicative of the biotechnological potential of a specific organism.

Interest in the study of marine sponges and their associated microbiomes has increased both for ecological reasons and for their great biotechnological potential. In consideration of the possible microbial origin and the sustainable supply of compounds, the cultivation of sponge-associated microorganisms could be the most direct method for the large-scale production of bioactive compounds. However, many specialized metabolite biosynthetic gene clusters are not expressed under conventional laboratory culture conditions (Rutledge and Challis 2015). Thus, activating these silent metabolite-producing pathways is a key challenge for the discovery of novel natural microbial products. Several methods have been developed to trigger the expression of such cryptic pathway-specific genes (Wang et al. 2015), including supplementing the fermentation media with chemicals, such as antibiotics, *N-acetyl glucosamine* (*GlcNAc*) and specific chemical compounds. Alternatively, mutations have been induced in genes encoding RNA polymerase (RNAP), S12 and other ribosomal proteins (Hosaka et al. 2009; Weber et al. 2015).

The Beibu Gulf, located in the northwest of the South China Sea, is a semi-closed gulf. An important geographical feature is plenty of estuaries, from which the river can bring in abundant natural nutrients for fish, sponge and reef growth (Chen et al. 2009). Previous studies on the Beibu Gulf mainly focused on hydrographical and sedimentological properties, or fishery resource management, even regarding marine sponges as a fouling organism (Yan et al. 2006; Zheng et al. 2012). In fact, the Beibu Gulf of South China Sea has become an important source region of marine natural compounds (Qian et al. 2015). However, because of a lack of information about dominant sponge species and associated microorganisms, the relevant resources of genes and natural products have yet to be comprehensively reported. In this study, a total of 49 sponge species from the Beibu Gulf of the South China Sea were sampled to investigate the diversity of the cultured bacterial. The antibacterial potential of associated actinobacteria, including the antibacterial activities and secondary metabolite-related genes, were screened and analyzed. Finally, the prominent sponge species and dominant actinobacteria with bioactivity were identified, which may prove useful in the discovery of novel natural compounds.

MATERIALS AND METHODS

Sample sites and sample collection

Forty-nine marine sponge samples were collected from four sites from the Beibu Gulf, South China Sea (Fig. 1). After collection, sterile sea water was used to rinse all samples three times in order to remove transient and loosely attached bacteria. Then all samples were kept in 25% glycerol in separate plastic sample collection tubes on ice and taken back to the laboratory as soon as possible. Most of the sponge tissues were used in isolating microbes, the partial tissue of each sponge sample was identified and the remaining samples were stored at -80°C for further analysis.

Isolation and cultivation of bacteria associated with marine sponges

Approximately 1 cm^3 sponge specimens were rinsed in sterile seawater and then thoroughly homogenized in a sterile mortar with 10 volumes of sterile seawater. The supernatant was 10-fold serially diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and subsequently plated out on varied media. A 100 μl aliquot of each dilution was plated onto the six selective isolation media (Table S1): marine agar 2216 plates (Difco, Detroit, MI, USA), ISP2 Agar (Shirling and Gottlieb 1966), YE Agar (Maddipati et al. 2011), M1 Agar (Mincer et al. 2002), R2A Agar (Leibniz Institute DSMZ, Braunschweig, Germany) and RH Agar (Difco), supplemented with cycloheximide (100 μg/ml), nystatin (5 μg/ml) and nalidixic acid (15 μg/ml). These plates for bacterial cultivation were prepared in triplicate and incubated at 28°C for 2–4 wk. All bacterial isolates with different colony morphotypes and microscopic appearances were selected, grown in pure cultures and stored in glycerol suspensions (20%, v/v) at –80°C. The 16S rDNA genes of selected representative isolates were amplified using the 27F and 1492R primers (Lane 1991).

PKS-KS and NRPS-A gene amplification from sponge-associated actinobacteria

All cultured sponge-associated actinobacteria isolated in this study were screened for PKS and NRPS genes using four sets of PCR primers, including DKF and DKR (Moffitt and Neilan 2003) for targeting PKS-KS sequences, KS1F1 and KS1R1 (Klein 2015) for targeting PKS-I sequences, KS2aF1 and KS2bR1 (Klein 2015) for targeting PKS-II sequences, and A3F and A7R (González

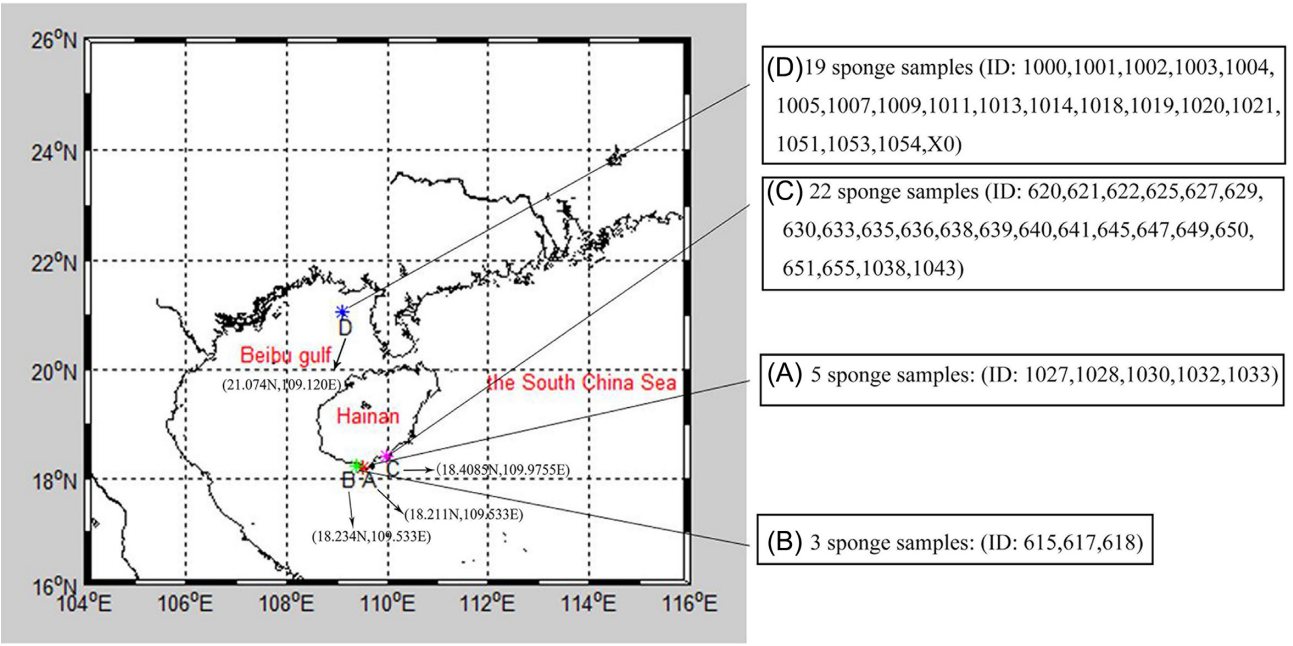

Figure 1 Sampling locations of marine sponges (A)-(D) from the Beibu Gulf, South China Sea.

et al. 2005) for targeting the adenylation domain in NRPS systems (Table 1). All amplified gene products were gel-purified and then cloned into pM19T (Takara Biotech), according to the manufacturer's instructions. The sequences were determined using M13+/− promoter primers and were subsequently further sequenced (Sangon Biotech). Products were visualized in 1% agarose gels stained with SYBR Green (Solarbio).

Phylogenetic analysis

The 16S rDNA gene sequences (~1500 bp) were amplified by Shanghai Life Technology Corporation. Near-complete 16S rDNA gene sequences were initially analyzed via BLAST at the National Center of Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) to aid in selecting the most closely related reference sequences. Sequence data were then edited using Clustal X1.83. The phylogenetic trees were constructed in neighbor-joining method using MEGA 6.0. Bootstrap analysis was performed with the Kimura 2-parameter model using 1000 replicates. The obtained 16S rDNA gene sequences, PKS gene sequences and NRPS gene sequences were deposited in GenBank under the accession numbers MG807483 to MG807605, MH780141 to MH780153 and MH537036 to MH537057, respectively.

Antibacterial activity assays

Initially, all strains were cultured in 50 ml eutrophic medium (Table S1) at 28°C for 3–4 wk and then 10μl supernatant of fermentation liquor was used for agar disc diffusion assay. However, over 95% actinobacteria species did not show any antibacterial activity. To promote secondary metabolite biosynthesis, strains were cultured in 50 ml of minimal medium (MM) at 28°C for 3–4 wk, after which the antibacterial activities of the extracts were determined using an agar disc diffusion assay. Six typical pathogenic organisms, including gram-positive (*Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633) and gram-negative bacteria (*Escherichia coli* ATCC 25 922, *Pseudomonas fluorescens* ATCC 27 853, *Vibrio alginolyticus* ATCC 33 787 and *Vibrio splendidus* ATCC 33 125) were tested for potential antimicrobial activity. These pure isolates were inoculated into 10 ml of liquid 2216E media and incubated at 28°C. After reaching the late stage of log phase, a 1 ml fraction of each culture was transferred to an Eppendorf tube and centrifuged at 12 000 r/min for 5 min. Subsequently, the resulting supernatants were used to saturate sterilized paper discs (Whatman, 6 mm) that were placed onto the surfaces of agar plates that were preinoculated with the indicator microorganisms. Finally, the diameter of the inhibition zone around each paper disc was measured.

RESULTS

Diversity of culturable bacteria associated with marine sponges

A total of 49 marine sponge species isolated from Beibu Gulf were identified with signature spicule types for each genus. Among them, 40 sponges from 16 genera were identified, and for nine sponge species it was difficult to identify their sponge class (Table S2). Of the four sampling sites, marine sponges from Lingshui and Weizhoudao had higher microbial biomass and diversity than other sampling sites (Fig. 2). *Haliclona* sp. was the prominent microbial sponge host, from which nearly 32% of total bacterial strains were obtained (Fig. 2), followed by *Callyspongia* sp. (11%), *Leucaltis* sp. (7%), *Desmacella* sp. (6%), unidentified sponge 615 (3%) and other sponges. Moreover, the marine sponge *Haliclona* sp. was also a remarkable Actinobacteria source. Thirty-two Actinobacteria isolates were obtained from *Haliclona* sp., including 12 isolates from the sponge species in Weizhoudao, 15 isolates from Lingshui and five isolates from Dadonghai (Fig. 2).

Table 1. Primers used in amplifying 16S rDNA sequences, PKS-KS and NRPS-A genes of sponge symbiotic microorganisms.

Primer	Target sequence	Sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)	Reference
27F	16S rDNA gene	AGA GTTTGA TCM TGG CTC AG	~1400	55	(Lane 1991)
1492R		GGY TAC CTT GTT ACG ACT T			
DKF	PKS clusters	GTGCCGGTNCCRTGNGYYTC	~700	52	(Moffitt and Neilan 2001)
DKR		GCGATGGAYCCNCARCARYG			
KS1F1	Type I KS domains	ATG GAY CCS CAR CAR CGB CT	~700	60	(Klein 2015)
KS1R1		GCY TCG ATS GGR TCN CCS A			
KS2aF1	Type II KS clusters	TSG CST GYT TCG AYG CSA T	~1070	60	(Klein 2015)
KS2bR1		GCR TAG AAC CAS GCG AWS GAC			
A3F	NRPS A denylation domains	GCSTACSYSATSTACACSTCSGG	~700	60	(González et al. 2005)
A7R		SASGTCVCCSGTSCGGTAS			

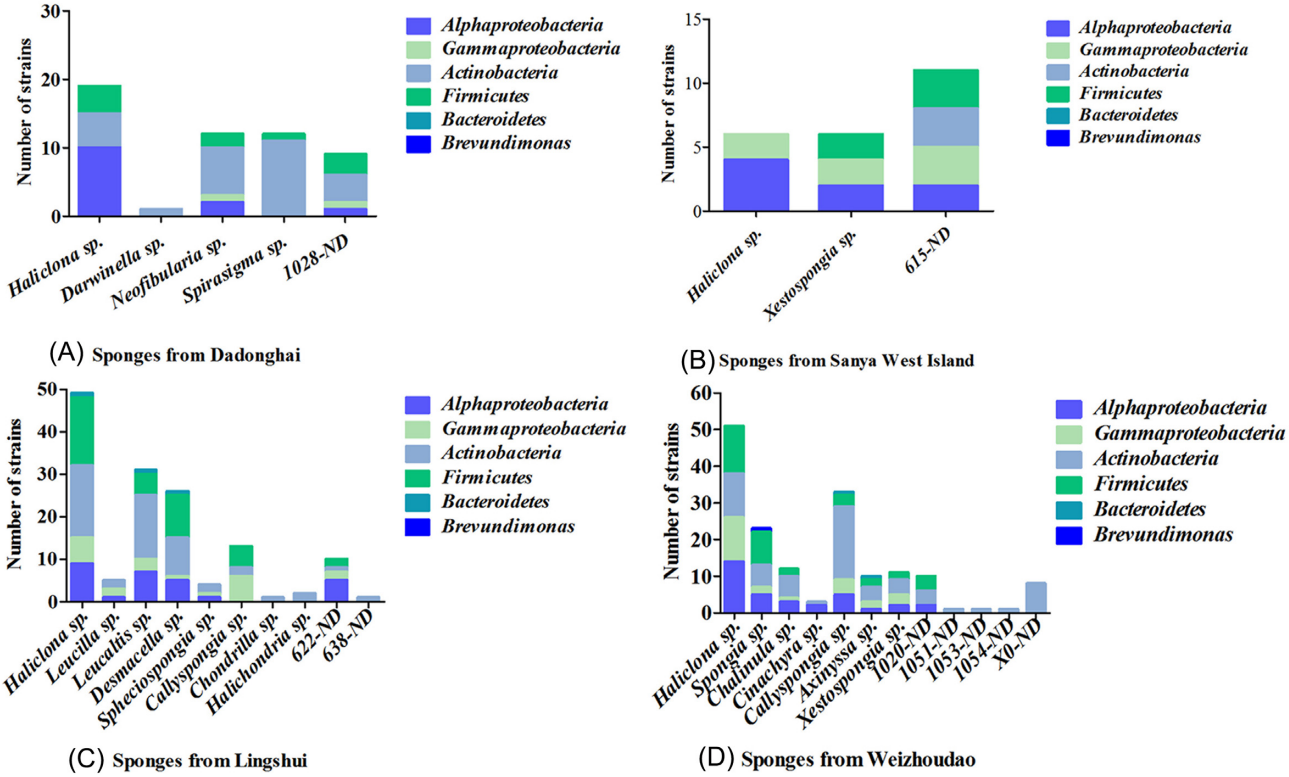

Figure 2 The distribution of sponge-associated bacterial strains in different marine sponges from the Beibu Gulf, South China Sea.

The 16S rDNA gene sequence analysis revealed that 363 bacterial isolates from sponges belonged to six phyla, including Proteobacteria (85 α-Proteobacteria strains and 59 γ-Proteobacteria strains), Actinobacteria (123 strains), Firmicutes (90 strains), Bacteroidetes (five strains) and Brevundimonas (one strain) (Fig. 3A). Of them, 123 actinobacterial isolates were clustered into 21 genera, including *Microbacterium* (37 species), *Pseudonocardia* (22), *Streptomyces* (13), *Kocuria* (10), *Aeromicrobium* (7), *Brachybacterium* (7), *Nocardiopsis* (4), *Micrococcus* (3), *Isoptericola* (2), *Brevibacterium* (3), *Mycobacterium* (3), *Corynebacterium* (2), *Tsukamurella* (2), *Citricoccus* (1), *Pseudokineococcus* (1), *Janibacter* (1), *Gordonia* (1), *Agromyces* (1), *Arthrobacter* (1), *Rhodococcus* (1) and *Dietzia* (1) (Fig. 3B). Most actinobacterial strains had higher 16S rDNA gene similarities to those present in GenBank. Only strain 1018–57, belonging to the genus *Corynebacterium*, exhibited a lower similarity than 97% (Table 2). Six culture media had different influences on the number and diversity of Actinobacteria recovered from marine sponges. The largest number of Actinobacteria strains were observed in modified R2A media for isolating 40 strains. By contrast, the traditional ISP2 media was more effective at culturing diverse Actinobacteria from 14 genera (Fig. S1). Interestingly, some actinobacterial strains grew exclusively on one medium, including isolates from the genera *Agromyces*, *Citricoccus* and *Mycobacterium* on R2A medium. By contrast, *Dietzia*, *Janibacter* and *Pseudokineococcus* species only grew on ISP2 medium.

Antimicrobial bioassay of sponge-associated cultivable actinobacteria

The potential to produce compounds with antimicrobial activity against the tested gram-negative (*E. coli*, *P. fluorescens*, *V. alginolyticus* and *V. splendidus*) and the gram-positive bacteria (*S. aureus* and *B. subtilis*) was evaluated for each actinobacterial strain via paper disk assay. In total, 92 out of 123 isolates

Table 2. Sponge-associated actinobacteria with moderate to strong antibacterial activities and PKS-KS/NRPS-A genes in this study.[a]

Genus	Isolates ID	Accession number	Medi-um	The closest bacteria in database	Simil -arities	Antibacterial activity against indicator strains[b]						Possible second metabolic related gene cluster[c]			
						Gram-positive bacteria			Gram-negative bacteria						
						S*	B*	E*	P*	V1*	V2*	PKSI	PKSII	NRPS	Hybrid**
Aeromicrobium	1011–16	MG807551	R2A	*A. kwangyangens* (EF693740.1)	99%	—	++	—	+++	+++	+	—	—	+	—
	1028–24	MG807576	R2A	*A. alkaliterrae* (NR_04 3207.1)	99%	+	+++	—	+++	+++	+	—	—	+	—
	1038–2.2	MG807592	R2A	*A. alkaliterrae* (NR_04 3207.1)	99%	—	++	—	++	++	+	—	—	—	—
	620–46.2	MG807492	YE	*A. alkaliterrae* (NR_04 3207.1)	99%	—	++	—	+	—	+	—	—	—	—
	X0–12.1	MG807598	ISP2	*A. alkaliterrae* (NR_04 3207.1)	99%	—	+++	—	+++	—	—	—	—	—	—
Agromyces	1001–6.1	MG807531	R2A	*A. indicus* (NR_108 908.1)	99%	+	+	—	+	+	—	—	—	—	—
Brachybacterium	1001–22	MG807532	M1	*B. paraconglomeratum* (JQ712514.1)	99%	—	+	—	+	++	+	—	—	—	—
	651–12	MG807527	ISP2	*B. paraconglomeratum* (NR_02 5502.1)	99%	—	++	—	+	+++	+	—	—	—	—
	1028–28	MG807577	R2A	*B. paraconglomeratum* (NR_02 5502.1)	100%	—	+	—	+	+	—	—	—	—	—
	620–38	MG807490	R2A	*B. paraconglomeratum* (JQ712514.1)	99%	—	++	—	+	++	++	—	—	—	—
	620–57	MG807498	YE	*B. tyrofermentans* (KC798059.1)	100%	—	—	—	++	++	—	—	—	+	—
	X0–14	MG807599	ISP2	*B. paraconglomeratum* (HQ202848.1)	99%	—	++	—	+	+	+	—	—	—	—
Corynebacterium	1018–35	MG807553	ISP2	*C. casei* (NR_122 062.1)	99%	+	++	—	—	++	+	+	—	—	—
	1018–57	MG807554	M1	*C. casei* (NR_122 062.1)	96.8%	+	—	—	+	++	+	+	—	—	—
Janibacter	620–4	MG807487	ISP2	*J. cremeus* (NR_114 380.1)	99%	+	++	++	+	++	++	+	—	—	—
Kocuria	635–8	MG807507	2216E	*K. sediminis* (KJ575013.1)	99%	—	—	—	++	++	+	—	—	—	—
	1019–33	MG807558	R2A	*K. polaris* (KM186612.1)	99%	—	+	—	+	++	+	—	—	—	—
	1028–10	MG807575	M1	*K. palustris* (LC020219.1)	100%	—	++	—	+	++	-	+	—	—	—
	620–1	MG807486	M1	*K. palustris* (KF424687.1)	100%	—	—	—	+	+	+++	—	—	—	—
	655–6.2	MG807528	ISP2	*K. palustris* (LC020219.1)	100%	—	+++	—	+++	+++	—	—	—	—	—
	X0–23	MG807604	YE	*K. flava* (JX007971.1)	99%	—	++	—	++	++	+	—	—	—	—
Microbacterium	1001–4.1	MG807530	R2A	*M. trichothecenolyticum* (JQ689178.1)	100%	—	++	—	—	++	+	—	—	—	—
	1007–29	MG807542	R2A	*M. trichothecenolyticum* (KJ631291.1)	100%	—	+	—	++	+	—	—	—	—	—
	1007–30	MG807543	R2A	*M. trichothecenolyticum* (JQ689178.1)	100%	—	+++	—	+++	+++	++	—	—	—	—
	1027–10	MG807571	M1	*M. oxydans* (DQ350825.1)	99%	—	—	—	++	++	+	—	—	—	—
	1027–11	MG807572	M1	*M. Paraoxydans* (KP064030.1)	99%	—	+	—	++	++	—	—	—	—	—
	625–25.2	MG807503	R2A	*M. aquimaris* (AM778450.1)	99%	—	+	—	++	++	+	—	—	—	—
	635–17	MG807508	2216E	*M. Arthrosphaerae* (JQ689174.1)	99%	—	+++	—	+++	+++	+	+	—	—	—
	635–23	MG807509	2216E	*M. trichotecenolyticum* (EU714362.1)	100%	+	+++	—	++	+++	+	—	—	—	—
	635–5.1	MG807505	YE	*M. trichothecenolyticum* (JQ689178.1)	100%	—	++	—	+++	+++	—	—	—	—	—

Table 2. Continued

Genus	Isolates ID	Accession number	Medi-um	The closest bacteria in database	Simil -arities	Antibacterial activity against indicator strains[b]						Possible second metabolic related gene cluster[c]			
						Gram-positive bacteria			Gram-negative bacteria						
						S*	B*	E*	P*	V1*	V2*	PKSI	PKSII	NRPS	Hybrid**
	639–5	MG807511	R2A	*M. paraoxydans* (KJ854553.1)	99%	+	++	—	++	++	+	—	—	—	—
	645–6	MG807512	R2A	*M. paraoxydans* (KM019860.1)	100%	+	++	—	++	++	+	—	+	—	—
	1020–16.2	MG807566	R2A	*M. pumilum* (KF876859.1)	99%	—	+++	—	+	—	+	—	—	—	—
	620–56	MG807497	YE	*M. chocolatum* (JX007959.1)	100%	—	—	—	+	++	++	—	—	—	—
	650–22	MG807523	ISP2	*M. esteraromaticum* (JN128279.1)	100%	+	+++	—	+	++	—	—	—	—	—
Nocardiopsis	650–36	MG807524	M1	*N. Terrae* (KC493982.1)	99%	++	++	++	—	++	++	+	—	—	—
	650–7	MG807520	YE	*N. dassonvillei* (JN253591.1)	100%	+	+	—	+	+++	—	—	—	—	—
Pseudonocardia	1043–2	MG807593	R2A	*P. carboxydivorans* (KP025716.1)	100%	—	+	—	+	+	—	—	—	+	—
	1004–13	MG807536	2216E	*P. carboxydivorans* (KC577579.1)	100%	—	+	—	+	+	—	—	—	—	—
	1009–19	MG807544	YE	*P. carboxydivorans* (KC577579.1)	99%	+	+	—	+	—	—	—	—	+	—
	1021–1	MG807569	ISP2	*P. carboxydivorans* (KC577579.1)	100%	+	+	—	+	++	—	—	—	—	—
	1019–26	MG807557	R2A	*P. carboxydivorans* (KC577579.1)	100%	—	—	—	+	++	—	+	—	+	+
	1019–39	MG807560	ISP2	*P. carboxydivorans* (FJ547123.1)	99%	+	—	—	+	+	+	+	—	+	+
	1032–8	MG807580	ISP2	*P. carboxydivorans* (KC577579.1)	100%	—	—	—	+	++	+	—	—	—	—
	1033–35	MG807588	R2A	*P. carboxydivorans* (KC577579.1)	99%	+	—	—	+	+	+	—	—	+	—
	1033–36	MG807589	R2A	*P. alni* (NR_119 240.1)	99%	+	+	—	+	+	—	—	—	+	—
Rhodococcus	650–4.3	MG807519	YE	*R. equi* (KF312643.1)	100%	+	+++	—	+++	+++	+	—	—	—	—
Streptomyces	1009–26.2	MG807546	R2A	*S. olivaceus* (KM370070.1)	100%	+	+	—	++	++	—	—	—	+	—
	620–52.1	MG807493	YE	*S. omiyaensis* (EU741148.1)	99%	+	—	—	+	+	—	—	—	—	—
	620–52.2	MG807494	YE	*S. omiyaensis* (EU741141.1)	99%	+	+	—	—	+	—	+	—	—	—
	650–1	MG807517	YE	*S. albidoflavus* (LN626361.1)	100%	+	+	—	+	++	—	+	—	—	—

[a]Actinobacteria listed in this table showed moderate to strong antibacterial activities.
[b]-, no activity; +, weak activity (8–10 mm); ++, good activity (10–14.5 mm); +++, excellent activity (>14.5 mm).
[c]-, negative; +, positive.
*S, *S. aureus*; B, *B. subtilis*; E, *E. coli*; P, *P. fluorescens*; V1, *V. alginolyticus*; V2, *V. splendidus*.
**hybrid PKS/NRPS gene.

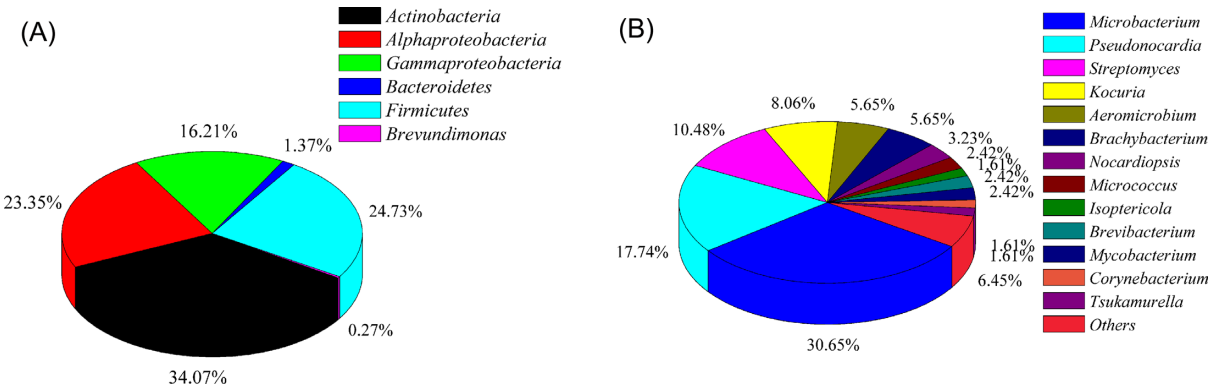

Figure 3 The relative abundance of bacterial phylum (A) and Actinobacteria genus (B) isolated from marine sponges.

exhibited antibacterial activity against at least one tested bacterium (Table S3). In this study, 51 strains displayed moderate to strong antimicrobial activity, which was defined as the ability to inhibit more than half of the tested strains (Table 2). Among these isolates, 22 isolates were observed against four of the test bacteria, which included members of the genera *Microbacterium*, *Pseudonocardia*, *Bachybacterium*, *Streptomyces*, *Aeromicrobium*, *Corynebacterium*, *Kocuria*, *Nocardiopsis* and *Agromyces*. Five actinobacterial isolates, belonging to the genera *Microbacterium*, *Nocardiopsis*, *Janibacter* and *Rhodococcus*, showed an attenuated growth effect for more than five test bacteria. The most broad-spectrum antibacterial activity was observed for one rare strain (at least in this study), *Janibacter* sp. 620–4, which exhibited activity against all the test strains. Comparison analysis of antimicrobial activity demonstrated that those with the most significant activities were members of the *Microbacterium*, *Aeromicrobium, Kocuria, Rhodococcus, Nocardiopsis* and *Brachybacterium* genera. Most notably, all seven isolates from the genus *Brachybacterium*, of which five were highly similar to *B. paraconglomeratum* and two had 99% similarity with *B. tyrofermentans*, showed more than one kind of antimicrobial activity. In particular, *Brachybacterium* strain 651–12 exhibited strong activity against *V. alginolyticus*. Additionally, some strains exhibited the greatest antagonistic activities against some specific test bacterium, such as *Microbacterium* X0–20 against *B. subtilis*, and *Microbacterium* 1002–50 against *P. fluorescens*.

Combining marine sponge host with bacterial distribution, the class Demonspongiae, especially the genus *Haliclona*, were the richest sources of diverse actinobacterial species with antimicrobial activities. Among 32 Actinobacterial strains from *Haliclona* sp., 22 isolates showed obvious bioactivities and belonged to the genera *Microbacterium*, *Brachybacterium*, *Pseudonocardia*, *Dietzia*, *Agromyces*, *Arthrobacter* and *Kocuria* (Table S3). Sponges of the genera *Callyspongia*, *Desmacella* and *Neofibularia* were also important members of the class Demonspongiae, possessing bioactive actinobacterial species. Although microbial diversity and richness associated with sponge was much lower than that of the class Demonspongiae, the sponge genus *Leucaltis* belonging to class Calcarea was the prominent Actinobacteria habitat, with 70% of total isolates (10/14) showing obvious antimicrobial activities.

Analysis of the PKS-KS and NRPS-A domains of sponge-associated Actinobacteria

According to the BLAST results for the PKS-KS and NRPS-A domain amino acid sequences, 34 isolates could yield sequence-verified gene products associated with one or two targeted domain types (Fig. S2). More specifically, 12 isolates possessed PKS-KS domains and 22 strains had NRPS-A domains. Seven actinobacterial strains harbored hybrid NRPS-PKS gene clusters, including *Tsukamurella* sp. X0–39 and *Microbacterium* sp. 1032–8.1. Genus *Pseudonocardia* were the most prominent genera with varied and rich secondary metabolic-related genes with 55% (12/22) (Fig. 4A). In addition, those genera containing relatively few strains had a fairly high proportion of PKS or NRPS genes, such as *Aeromicrobium* (3/7), *Tsukamurella* (2/2) and *Nocardiopsis* (1/4). The heatmap analysis of PKS and NRPS sequence similarity showed that the gene fragments found in 34 isolates were all different between each other (Table S4, Fig S3).

By the conjoint screening for antimicrobial activities and functional genes, the majority of isolates with PKS or NRPS genes showed antimicrobial activities to some extent (Fig. 4B). Only three isolates (3/34) had no detectable activities in this study. By contrast, 68 strains out of 123 isolates with antimicrobial activities had no secondary metabolites-related genes amplified with the primers used in this study. In particular, 27 isolates within the genera *Microbacterium* (17 isolates), *Kocuria* (seven isolates) and *Aeromicrobium* (three isolates) that showed significant antimicrobial activities had no PKS-KS or NRPS-A genes detected in this study.

DISCUSSION

Sponge-microbial associations that synthesize clinically significant bioactive compounds have been discovered from geographically different regions, such as the Great Barrier Reef of Australia, the Mediterranean Sea, Indonesia, Papua New Guinea, the Indo-Pacific region and the South China Sea (Weber et al. 2015). In this study, cultured bacterial isolates from 49 sponge species along the Beibu Gulf of the South China Sea were screened and evaluated for their antibacterial potential. The abundant sponge species confirmed that the typical semi-enclosed bay of the Beibu Gulf provided a natural geographical advantage for the growth of sponges. Sponge genus *Haliclona* was the most widespread among four sampling sites, followed by genera *Callyspongia* from Lingshui and Weizhoudao. Sponge Demonspongiae generally represent an ecological niche that harbors great microbial diversity and metabolic potential (Ise 2017), in which the genera *Haliclona*, *Callyspongia* and *Desmacella* were the characteristic holobiont for each sampling site. In particular, *Haliclona* sp. harbored the greatest microbial diversity in this study, which was a common temperate sponge and appeared to be a promising source of lead compounds, with as many as

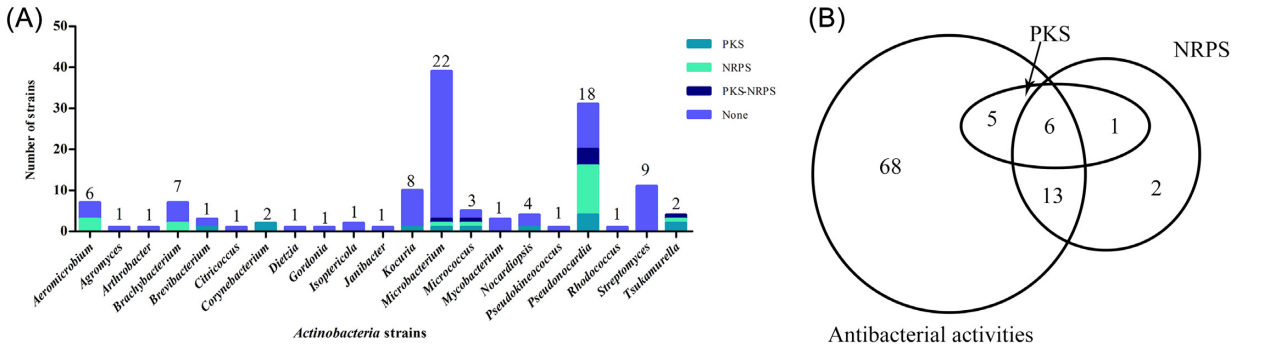

Figure 4. The distribution of functional genes and antibacterial activities of actinobacteria from marine sponges. (**A**) The numbers of actinobacterial genus with secondary metabolic related genera (clusters) or antibacterial activities; (**B**) Venn diagrams of shared sponge-associated species with secondary metabolic related genes (clusters) and antibacterial activities (cfus).

190 compounds of various chemical classes and functions having been reported (Viegelmann et al. 2014). Further studies have confirmed the role of microorganisms in the production of bioactive compounds in *Haliclona* sp. (Hoppers et al. 2015).

Although from 1976 to present only 200 compounds have been derived from Calcisponge and their associates (Roué et al. 2012), our results revealed the potential of the genus *Leucaltis*, class Calcarea. Due to much thinner mesohyl of the calcareous body, most sponges have a relatively lower microbial abundance, thus have remained a largely overlooked class in contrast to the sponge class Demonspongiae. Sponges of the class Calcarea include five orders and 24 families, and only one family (Leucetidae) has been identified as a source of a pharmacologically significant bioactive compound, noribosomal cyclic peptide leucamide A, which could be of microbial origin (Viegelmann et al. 2014). Flemer et al. observed that 15% of the isolates from *Leucosolenia* sp. (family Leucosoleniidae, class Calcarea) also showed activity against the tested fungal strains (Flemer et al. 2012). In this study, 71% of actinobacterial isolates, which were exclusively from *Leucaltis*, demonstrated clear activity against at least one test strain. It highlights the potential of Calcisponge as a microbial source for novel metabolites. Synchronously, this could be the first report of microbial isolates from sponges of the genera *Desmacella*, *Neofibularia*, *Spirasigma*, *Halichondria*, *Spheciospongia* and *Chondrilla* inhibiting the growth of microbes.

One investigation of the global sponge microbiome showed that only the phylum Proteobacteria (especially α- and γ-proteobacteria) was dominant in most sponge species, with Chloroflexi, Cyanobacteria and Crenarchaeota occasionally reaching high relative abundances (~10%) (Thomas et al. 2016). This phylum-level trend was also observed for isolates with dominant α- and γ-proteobacteria in this study. In addition, in total 52 *Bacillus* strains within Firmicutes were isolated in this study, which have been reported concerning their bioactivities (Vijayalakshmi, Rajasekar and Mohankumar 2017; Natesh, Arumugam and Karanam 2018). Although the two groups had high abundance and possible antibacterial activities, the bioactive potential was mainly aimed at actinomycetes strains because of their important representatives of marine resources of leading compounds (Karuppiah et al. 2015). The distribution of clinically active compounds obtained from bacteria indicated that 50% of the novel therapeutic compounds could derive from members of the phylum Actinobacteria (Dharmaraj 2010). A total of 123 actinomycete species were classified into 21 bacterial genera, most of which clustered together in a phylogenetic tree and were distinct from those bacterial species derived from other sources. The *Microbacterium-Pseudonocardia* group was the major cultivable actinobacteria, followed by *Streptomyces*, *Kocuria, Aeromicrobium* and other genera. It was noticeable that the genera *Agromyces*, *Brevibacterium*, *Citricoccus*, *Isoptericola* and *Pseudokineococcus* were isolated from marine sponges for the first time (Graça et al. 2015).

More than 80% of actinobacterial isolates showed bioactivity potential with antibacterial activities or functional genes. Among them, 74% of actinobacteria showed antagonism against at least one test bacterium, with 40% of isolates exhibiting moderate to strong activities. Previously, the genus *Streptomyces* had the largest number of species generating leading compounds from many various sponge species (Khan et al. 2011); conversely, few *Microbacterium* species for secondary metabolism have been reported (Abdelmohsen, Bayer and Hentschel 2014). Four glycoglycerolipids and one diphosphatidylglycerol with antitumor activities have been isolated from sponge-associated *Microbacterium* sp. (Wicke et al. 2000). In this study, the *Microbacterium* genus was actually the most active genus, with diverse isolates and significant antibacterial activities. Different isolating protocols or a low-nutrient composition supplemented with *GlcNAC* could improve the discovery of *Microbacterium* genus for antibacterial abilities. Members of the genus *Nocardiopsis* were the second biotechnologically important group of actinomycetes in this investigation, most of which primarily produce polyketides, quinoline alkaloids, proteins, thiopeptides and other bioactive compounds (Bennur et al. 2016). Compared with other actinobacteria, *Bachybacterium* genus also showed promising antibacterial potential, although no antibacterial activity had been previously detected in other studies.

The majority of actinobacteria genera, such as *Arthrobacter*, *Agromyces*, *Citricoccus*, *Corynebacterium*, *Dietzia*, *Gordonia*, *Janibacter*, *Micrococcus*, *Nocardiopsis*, *Pseudokineococcus*, *Rhodococcus* and *Tsukamurella*, were present in lower abundance and diversity, whereas these genera exhibited a high percentage of antimicrobial activity. Different selective media, which were used to activate silent secondary metabolic pathway, could contribute to the positive screening of antibacterial activities. The use of nutrient-poor culture media is a powerful tool for promoting marine microorganisms to produce bioactive compounds with the aim of antagonizing the growth of other microorganisms (Connon and Giovannoni 2002). Rigali et al. (2008) also showed that the accumulation of *GlcNAc* during cell-wall hydrolysis in famine condition triggers development and antibiotic production of *Streptomycetes*. A high concentration of *GlcNAC* in oligo-culture perhaps mimicked the accumulation of *GlcNAC* after autolytic degradation of the vegetative mycelium. This

result suggested that antibacterial activities triggering effect of *GlcNAc* was common in actinobacteria species, although not universal, at least under the conditions we studied.

The genomic screening of bioactive potential was a useful method to identify new bioactive molecules. Among these functional gene clusters, the identification of complex PKS and NRPS has been a powerful strategy to reveal the potential of the ability of strains to produce bioactive compounds (Agustina et al. 2016). Finally, 45/123 isolates were observed to harbor PKS-KS, NRPS-A or PKS-NRPS domains and the vast majority of Actinomyces with functional genes also showed antimicrobial activities. Nevertheless, 53% (49/92) of positive actinobacterial strains for bioactivity were not detected related PKS or NRPS genes. This observation may indicate their ability to produce different types of bioactive compounds. Therefore, the prediction of secondary metabolites by only detecting biosynthetic gene clusters could lead to a decreased discovery of novel bioactive compounds (Vartoukian et al. 2016).

The isolation of strains performed in this study enabled the recovery of abundant and diverse bacterial strains. Although variations in media composition have been used to increase the recovery of Actinobacteria, the number of bioactive strains were still limited to relatively rich sponge-associated Actinobacteria resources. In future, more culturing projects or diverse sponge resources could increase the probability of getting novel bioactive strains. More importantly, activating many cryptic pathways for secondary metabolite of actinobacteria by different methods could improve natural products from those strains isolated.

CONCLUSION

The present study showed diverse culturable microorganisms and their antibacterial potential from 49 marine sponge species along the Beibu Gulf, South China Sea. Sponge class Demonspongiae, such as genus *Haliclona* sp., *Callyspongia* sp. and *Desmacella* sp., was the dominant microbial host. Meanwhile, calcareous sponge that was usually overlooked was also a promising bioactive holobiont, especially *Leucaltis* genus. In total, 363 isolates clustered into six bacterial phyla, including 123 Actinobacteria strains within 21 genera. Among actinobacterial genus, *Microbacterium* and *Pseudonocardia* were the most bioactive genera with a strong and broad spectrum of antibacterial activities. The minimal medium was efficient enough to activate bioactive substance production, 92 out of 123 actinobacterial isolates showing antimicrobial activities and 51 displaying moderate to strong antimicrobial activities. Of actinobacterial isolates associated with sponges, ~37% contained PKS-KS or NRPS-A gene clusters, revealing the potential discovery of natural products.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflicts of interest. None declared.

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