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ORIGINAL ARTICLE

Antimicrobial activity and diversity of bacteria associated with Taiwanese marine sponge *Theonella swinhoei*

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Abstract

Marine sponges often rely on other epiphytes for protection from harmful predators. To understand the diversity and antimicrobial activity present among epiphytic bacteria isolated from marine sponge. We used both the 16S rRNA tag pyrosequencing method and the culture-based method to investigate the bacterial communities of *Theonella swinhoei* collected off the shore of southern Taiwan. Eight-hundred and eighteen operational taxonomic units (OTUs; 97% sequence similarity) were identified from 23,700 sponge-derived sequence tags. The bacteria associated with *T. swinhoei* were found to be highly diverse—as many as 12 different phyla of bacteria were identified. However, in terms of population evenness, the community was dominated by two phyla—Acidobacteria (71.54%) and Chloroflexi (19.60%). A total of 700 bacterial strains were isolated and cultured from samples of the sponge *T. swinhoei*. Within these culturable strains, only 12% were Actinomycetes. Despite the low percentage of Actinobacteria from the samples, among the 51 strains of culturable bacteria that showed high antimicrobial activity, a great majority (62%) were Actinomycetes (30 strains of *Streptomyces* and 1 strain each of *Micromonospora* and *Brevibacterium*). The remaining isolates that produced antimicrobial compounds were Gammaproteobacteria (10 strains of *Pseudoalteromonas*) and Firmicutes (8 and 1 strains of *Bacillus* and *Paenibacillus*, respectively). We speculated that many more Actinomycetes are yet to be isolated from *T. swinhoei* microbiota. Advanced techniques, such as high-throughput culture and culturome, should allow the isolation and purification of these medically important groups of bacteria from sponge.

Keywords Sponge-associated bacteria · Antimicrobial activity · Cytotoxic activity · Metagenomic analysis · 454 pyrosequencing

Introduction

The ocean carries many microbes, most of which are attached to other marine organisms (Burgess et al. 1999; Nithyanand et al. 2011). Sponges host a large community of microorganisms, including various bacteria, fungi, unicellular algae, and archaea (Taylor et al. 2007; Webster and Taylor 2012). The biomass of microbes in some sponges can constitute up to 40–60% of the host's total biomass (Hill et al. 2006). Many of these microbial symbionts are photosynthetic, whereas others are nitrogen fixers and nitrifiers. They form a complex community to support the many nutritional, health, and defense needs of the sponge (Bayer et al. 2008; Flatt et al. 2005; Hoffmann et al. 2009; Osinga et al. 2001; Thomas et al. 2010; Wilkinson and Fay 1979; Wilkinson et al. 1981). Although most of these microbes are yet to be cultured, recent massive parallel DNA sequencing studies have provided new insight into bacterial communities in sponges. Sponges are associated with more than 47 bacterial phyla (Reveillaud et al. 2014). Very often, genetically related sponges collected from very different geographical locations harbor a similar microbiota, suggesting the presence of specific associations between sponges and bacterial symbionts (Reveillaud et al. 2014; Thoms et al. 2003).

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Marine organisms may produce chemicals of great value (Leal et al. 2012; Mehbub et al. 2014). Accumulated evidence has shown that some natural products formerly attributed to being produced by the sponge host are actually produced by symbiotic bacteria (Unson and Faulkner 1993; Unson et al. 1994). Moreover, it has also been demonstrated that more than 80% of FDA-approved drugs or pharmaceutical agents in clinical trials isolated from marine organisms are predicted to originate from associated microbes (Gerwick and Moore 2012). Sponges are a primitive group of ocean animals belonging to the phylum Porifera. Many antimicrobial compounds have been isolated from sponge-associated microbes (Graca et al. 2013; Hentschel et al. 2001; Thomas et al. 2010; Unson and Faulkner 1993; Unson et al. 1994). Over the years, researchers have noticed that new species of different genera of Actinobacteria, commonly present in marine sponges, can produce novel antimicrobial compounds (Abdelmohsen et al. 2014). Graca et al. (2013) studied the activities of bacterial symbionts from the sponge *Erylus discophorus*, and reported that as many as 31% of the isolates could produce antimicrobial metabolites. Among the antimicrobial-producing bacteria, *Pseudovibrio*, *Vibrio*, and *Bacillus* are the three most common genera. On the other hand, *Pseudoalteromonas*, a member of the γ-Proteobacteria family, and many α-Proteobacteria are the most abundant bacteria that produce antimicrobial compounds from sponges *Aplysina aerophoba* and *Aplysina cavernicola* (Hentschel et al. 2001).

Marine sponge *Theonella swinhoei* (class Demospongiae, order Tetractinellida, family Theonellidae) is distributed throughout the Indo-West Pacific. Forty percent of the biomass of the sponge is contributed by microbes (Jin et al. 2014; Keren et al. 2015). Natural *T. swinhoei* sponges produce products such as bioactive polyketides (Bewley et al. 1996; Piel et al. 2004) and glycopeptides (Schmidt et al. 2000). The bacterial community inhabiting *T. swinhoei* has been studied using the clone library technique (Hentschel et al. 2002) and culture-based methods (Keren et al. 2015; Lavy et al. 2014). While these methods can identify the dominant members of the community, many of the low-abundance taxa in the community are likely to have been missed. Pyrosequencing DNA technology is an ultra-deep DNA sequencing method that provides a large number of DNA sequence reads in a single run. When this deep sequencing method is applied to detect 16S DNA in environmental samples, many low-abundance taxa can be identified. We analyzed the diversity of bacteria inhabiting *T. swinhoei* using the tagged 16S rRNA pyrosequencing DNA sequencing method, and isolated bacteria, with a particular focus on actinomycetes with antimicrobial activities from *T. swinhoei*.

Materials and methods

Sponge collection and processing

Specimens of sponge *T. swinhoei* were collected during February 2013 at the inlet of a nuclear power plant within Kenting National Park, southern Taiwan (21°94′42.60″N, 120°79′31.97″E) by hand at a depth of about 3–5 m below sea level. The samples were placed in sterile plastic bags in a cold water environment for transportation to the laboratory immediately after collection. Three pieces of sponge tissue (each approximately 0.5 g wet weight) from different parts of one sponge were cut with a sterile scalpel, thoroughly washed twice with filtered (0.22 μm) seawater, pooled together and homogenized with 5 ml filtered seawater in a sterile mortar, then transferred to a 50-ml centrifuge tube. The sponge homogenate prepared was used for bacterial community DNA extraction and sponge-associated bacteria cultivation.

Bacterial community DNA extraction and 16S rRNA gene tag sequencing

Total genomic DNA for two *T. swinhoei* samples, A and B, was extracted and purified using a GeneMark genomic DNA purification kit (GeneMark, Taipei, Taiwan) according to the manufacturer's protocol. PCR, amplicon purification, and quality assessment, as well as tag sequencing, were performed at Welgene Biotech (Taipei, Taiwan) following the protocols described previously (Hentschel et al. 2002; Lavy et al. 2014). Samples A and B are collected at the same place and the location distance between them is less than 1 m. Primer sets 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCG CGGCTGCTGGCAC-3′) were used to amplify the V1–V3 hypervariable regions of bacterial 16S rRNA genes. 16S rRNA gene tag sequencing was performed on a Roche 454 Life Science FLX pyrosequencer (454 Life Sciences, Barnford, CT, USA).

Pyrosequencing data handling and bioinformatics analysis

All trimming, clustering, and classification were performed using the Mothur software package (Reveillaud et al. 2014; Schloss et al. 2011, 2009). Prior to cluster analysis, sequences that had the low-quality reads (reads shorter than 200 bp and with an average quality score above 25) (Simister et al. 2012), had homopolymer longer than 8 bp, and contained chimeras identified using the chimer.uchime command in Mothur were trimmed from analysis (Schloss et al. 2011, 2009). The clean reads were then aligned against the SILVA database (release 119) (Pruesse et al. 2007) and clustered into operational taxonomic units (OTUs) at a 3% dissimilarity cutoff point based

on the furthest neighbor-clustering algorithm. The taxonomic position of each OTU was assigned from the SILVA database (Pruesse et al. 2007). The RDP-classifier was used to assign a phylotype for representative sequence from each OTU, with an 80% confidence threshold (Cole et al. 2005). Alpha and beta diversity metrics, including the Shannon-Weaver diversity index, the Chao1 richness estimator, and the abundance-based coverage estimator (ACE) as well as the Bray-Curtis dissimilarity, the Morisita-Horn dissimilarity, and the Jaccard dissimilarity, were also calculated using Mothur software. The coverage was estimated using program Nonpareil version 3.3, using alignment algorithm (Rodriguez-R and Konstantinidis 2014).

Sequences similarity search between 16S rRNA genes of isolates and OTUs as well as OTUs and SILVA database were conducted using stand-alone BLAST+ program (version 2.2.30) from NCBI (Camacho et al. 2009).

Sponge-associated bacteria cultivation

Seven different selective media, soil agar (SA), Gause modified agar I (GIA), actinomycete isolation agar (AIA), M1A agar (M1A), glucose-peptone-yeast extract agar (GPYA), Gause mineral agar (GHA), and peptone-yeast extract agar (PYA), were used to culture and isolate bacteria from the sponge homogenate. The compositions of these media were as follows: soil agar—1 ml soil extract (200 g soil mixed in 1 l 50% seawater, followed by boiling for 30 min), 1 ml vitamin complex solution (0.5 mg/ml calcium pantothenate, 0.5 mg/ml nicotinic acid, 0.05 mg/ml thiamin chloride, 0.05 mg/ml biotin), 0.05 g/l nalidixic acid, 0.025 g/l nystatin, and 20 g/l agar (Bredholdt et al. 2007); Gause modified agar I—20 g/l soluble starch, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l K_2HPO_4, 1 g/l KNO_3, 0.5 g/l NaCl, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.05 g/l nalidixic acid, 0.025 g/l nystatin, and 20 g/l agar (Terekhova et al. 1991); actinomycete isolation agar—22 g/l actinomycete isolation broth, 0.025 g/l nystatin, 0.05 g/l cycloheximide, and 20 g/l agar; M1A agar—10 g/l starch, 4 g/l yeast extract, 2 g/l peptone, 0.05 g/l nystatin, 0.05 g/l cycloheximide, and 20 g/l agar (Jensen et al. 2005); glucose-peptone-yeast extract agar—20 g/l glucose, 5 g/l peptone, 5 g/l yeast extract, 0.05 g/l streptomycin, and 20 g/l agar; Gause mineral agar—20 g/l soluble starch, 1 g/l KNO_3, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l K_2HPO_4, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.025 g/l nystatin, 0.05 g/l cycloheximide, and 20 g/l agar (Ivanitskaia et al. 1978); and peptone-yeast extract agar—5 g/l peptone, 5 g/l yeast extract, 0.025 g/l nystatin, 0.05 g/l cycloheximide, and 20 g/l agar. All the above media were supplemented with 50% sea water to increase the chance of isolating bacteria adapted to the marine environment. After plating of the sponge homogenate, the plates were incubated at 25 °C for 5 days to 2 weeks. Individual colonies were chosen and streaked onto fresh M1 agar (M1A without nystatin and cycloheximide) until pure cultures were obtained. For long-term preservation, cultures were grown at 25 °C in M1 broth and stored at − 80 °C in 20% glycerol solution.

Antimicrobial activity screening

Two screening steps, primary and secondary screening, were used to screen sponge isolates for antimicrobial activity. Primary screening was performed to select preliminary antimicrobial isolates using the agar block method (Chen et al. 2012; Stern et al. 2006) against a Gram-negative strain (*Vibrio parahaemolyticus*), a Gram-positive strain (*Staphylococcus aureus*), and a yeast (*Candida albicans*). Isolates that exhibited an inhibition zone > 1 mm in diameter against at least one test strain in primary screening were selected for secondary screening using the agar block method (Stern et al. 2006) against five test bacteria, namely *Escherichia coli*, *V. parahaemolyticus*, *Pseudomonas aeruginosa*, *S. aureus*, and *C. albicans*.

Test microorganisms were cultured as follows: *V. parahaemolyticus* (BCRC 10806) and *P. aeruginosa* (BCRC 10303) were cultured in marine broth at 25 °C for 24 h and maintained on marine agar; *C. albicans* (BCRC 22903) was cultured in YM broth at 30 °C for 48 h and maintained on YM agar; and *S. aureus* (ATCC 12600) and *E. coli* (ATCC 11775) were cultured in LB broth at 37 °C for 18 h and maintained on LB agar.

Crude extract preparation

Sponge isolates that exhibited antimicrobial activity were cultured in 500-ml flasks containing 100 ml M1 medium with 50% seawater. Flasks were incubated at 25 °C on a rotatory shaker at 150 rpm. After 5 days of incubation, the fermented broths were extracted twice with ethyl acetate (100 ml × 2). The solvent extracts were combined and evaporated to dryness under vacuum. The extracts obtained were weighed and stored at − 20 °C. The extracts were then used for cytotoxic activity assays.

Detection of cytotoxic activity

The cytotoxic activity of the crude extract was studied by MTT assays (Lu et al. 2009). The cell lines used were cancer cell lines MCF-7, MDA-MB-231, and T47-D from breast tumors, MOLT-4 and K-562 from leukemia, and DLD-1 and HCT-116 from colorectal cancer. All cell lines were purchased from the ATCC collection. Cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2.

Gene sequencing and phylogenetic analysis

Isolates that exhibited antimicrobial activity were identified by their 16S rRNA gene sequence. A 16S rRNA fragment of each strain was amplified using the universal 16S rRNA bacterial primer pair 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CTACGGCTACCTTGTTACGA-3′). Sequencing was performed by Tri-I Biotech (Taipei, Taiwan).

A neighbor-joining tree was constructed with Kimura's two-parameter model using MEGA software ver. 5.0 (Tamura et al. 2011). The statistical significance of the resultant tree topology was evaluated by bootstrap analysis, involving the construction of 1000 trees from randomly resampled data.

Nucleotide sequence accession numbers

The nucleotide sequences of isolates obtained in this study have been deposited in the GenBank database under accession numbers from MH311883 to MH311933. The raw pyrosequencing reads obtained in this study were deposited in the NCBI Sequence Read Archive (SRA) with the accession number SRP145405.

Results

Diversity of sponge-associated bacteria with pyrosequencing data

To investigate the diversity of bacteria associated with *T. swinhoei* at a deeper level, total DNA obtained from sponge extracts was used as a template for 16S rDNA tag pyrosequencing. A total of 27,548 raw gene sequence reads (approximately 13,179,125 bp) spanning the V1–V3 variable regions were obtained from two *T. swinhoei* samples, samples A and B. Table 1 presents a summary of the OTUs, Coverage, Shannon-Weaver and Simpson diversity indexes, and ACE and Chao1 indexes for richness. The high differences between OTUs and ACE or Chao1 suggested that further sequencing of the samples may reveal greater OTU richness. We found that although we obtained more reads and coverage from sample B, the diversity and richness in sample B were slightly higher than those of sample A.

After removing low-quality reads, we obtained 23,700 clean reads, 10,573 from sample A and 13,127 from sample B, with an average length of 485.24 and 488.70 bp, respectively. The 23,700 reads of bacterial communities of the sponge *T. swinhoei* could be classified into 12 phyla (Fig. 1a). The bacterial community structure of samples A and B varied little at the phylum level. Acidobacteria (71.54% of total reads), Chloroflexi (19.60%), Proteobacteria (2.62%), Planctomycetes (2.58%), Actinobacteria (1.70%), Cyanobacteria (0.56%), and Firmicutes (0.25%) were the seven major phyla. Thus, on the phylum level, more than 70% of the bacterial reads from *T. swinhoei* were affiliated with phylum Acidobacteria. This result is in line with the median value of the Shannon-Weaver diversity index. In addition to the phyla mentioned above, eight less-dominant bacteria, belonging to the phyla Bacteroidetes, Verrucomicrobia, Deferribacteres, and candidate phylum TM6 and TM7, were also identified from the sponge samples.

The clean reads were assigned to 818 unique OTUs (based on a 97% sequence identity), among which sample A contained 529 OTUs, sample B contained 524 OTUs. Only 235 OTUs (28.73% of total OTUs) were shared between samples A and B, but these OTUs include 95.97% total reads. To know the difference between two samples, the Bray-Curtis, Morisita-Horn, and Jaccard dissimilarity were calculated to describing the dissimilarity between two communities and their values were 0.114, 0.004, and 0.712714, respectively. The inconsistence between Jaccard index and two other indices is Jaccard index does not consider the abundance of species. Therefore, we can conclude that the difference in the microbial communities between two samples is small, but rare phylotypes between them are different.

Four-hundred and fifty-five OTUs (55.50% of the total OTUs) or 5529 reads (22.85% of the total reads) could be classified into 50 separate families. The inability to resolve the taxonomic ranking of these bacteria below the family level suggested that numerous bacteria associated with *T. swinhoei* are still unclassified in the literature or unknown. Caldilineaceae (19.19%) was the most abundant family detected from *T. swinhoei*, followed by Planctomycetaceae (2.49%), Coxiellaceae (0.26%), Anaerolineaceae (0.18%), Sinobacteraceae (0.18%), Lachnospiraceae (0.12%), and Rhodospirillaceae (0.12%) (Fig. 1b; Supplementary File 1).

The top 20 most abundant OTUs, which were responsible for 81% of the total sequences of the two sponge samples in this study, were compared with the GenBank nt database using blastn, and the results are listed in Table 2. Among the 20 most abundant OTUs, 10 were affiliated to Acidobacteria, 8 to Chloroflexi, and 1 to Actinobacteria and Planctomycetes.

Antimicrobial activity analysis

A total of 700 isolates (including 606 bacteria and 94 actinomycetes) were cultured from homogenates of the marine sponge *T. swinhoei*, collected at Nanwan Bay in southern Taiwan, using seven types of culture media. They were subsequently evaluated in terms of their antimicrobial activity using the agar block method in primary screening. Among the isolates, 15% (105), including 65 bacteria and 40 actinomycetes, were found to possess antimicrobial activity against at least one of the three test strains, *V. parahaemolyticus*, *S. aureus*, and *C. albicans* (Table 3). Isolates belonging to

Table 1 Summary of numbers of reads and OTUs, in addition to coverage and diversity estimators, of the 16S rRNA gene sequences of the bacterial community of marine sponge *Theonella swinhoei* (samples A and B)

Sample ID	Reads	OTUs	Ace	Chao1	Coverage	Shannon	Simpson
Sample A	10,573	529	1383	1067	0.98357	3.02	0.2295
Sample B	13,127	524	1140	863	0.99270	2.85	0.2411

actinomycetes showed a greater percentage of antimicrobial activity (42.55%) than bacteria (10.73%).

Fifty-one isolates (including 19 bacteria and 32 actinomycetes) for which an inhibition zone > 1 mm in diameter was observed against at least one test strain were selected for secondary antimicrobial screening against five pathogens, *S. aureus*, *E. coli*, *V. parahaemolyticus*, *P. aeruginosa*, and *C. albicans*, and the results are presented in Fig. 2a, b. Among the 51 isolates, 84.31% inhibited a Gram-positive bacterium (*S. aureus*), 54.90% inhibited Gram-negative bacteria (*E. coli*, *V. parahaemolyticus*, and/or *P. aeruginosa*), 39.22% inhibited a fungus (*C. albicans*), and 49.02% inhibited both Gram-positive and Gram-negative bacteria. Five isolates (9.80%), including four bacteria, PYAN1-2, PYAN10-2, PYAN10-21, and PYAN1-3, and one actinomycete, SAU1-231, inhibited more than three test strains. The percentage of susceptibility of

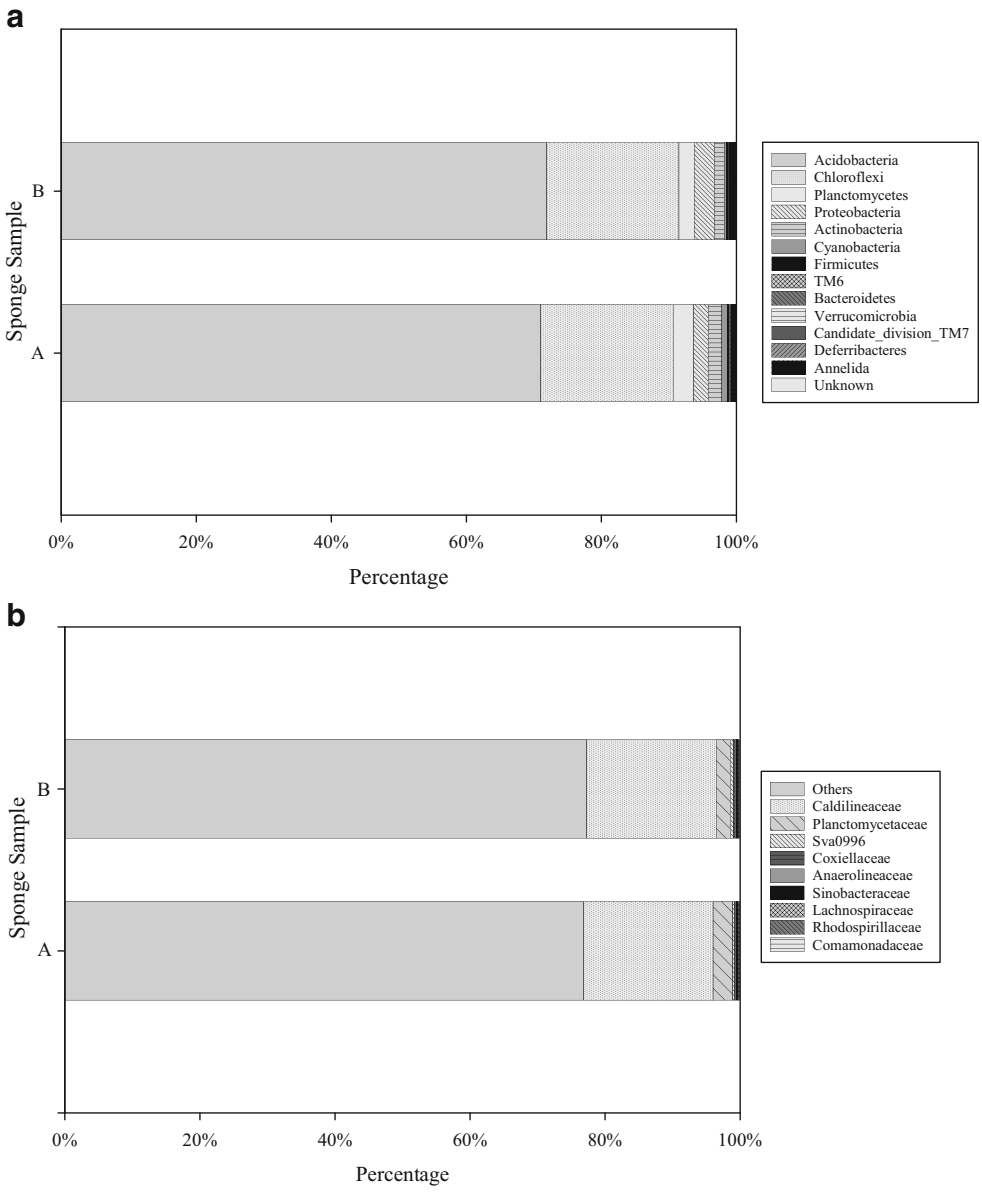


Fig. 1 Taxonomic diversity of the bacterial community associated with marine sponge *Theonella swinhoei* at the phylum level (samples A and B)

Table 2 BLAST analysis of the 20 most abundant OTUs in *Theonella swinhoei* samples A and B

OTU	Percentage of total sequence of sample (%)		Accession number	Description	%Identity
	Sample A	Sample B			
OTU1	46.77	47.52	FJ543132.1	Clone OP444 (Acidobacteria; Holophagae; TK85)	98%
OTU2	7.85	10.38	FJ269280.1	Clone XA2F04F (Acidobacteria; Holophagae)	98%
OTU12	3.23	3.01	FJ543132.1	Clone OP444 (Acidobacteria; Holophagae; TK85)	98%
OTU3	2.20	2.61	AY897114.1	Clone Dd-spT-A21(Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	98%
OTU22	2.04	1.94	GU118696.1	Clone Mfav_M05 (Acidobacteria; Holophagae; TK85)	98%
OTU5	1.83	1.88	FJ543133.1	Clone OP410 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	96%
OTU6	1.70	1.87	AY897114.1	Clone Dd-spT-A21 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	97%
OTU4	1.51	1.67	JN655343.1	Clone PO10-3-1_C24 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	98%
OTU14	1.68	1.47	FJ269280.1	Clone XA2F04F (Acidobacteria; Holophagae; TK85)	98%
OTU9	1.66	1.44	FJ543133.1	Clone OP410 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	96%
OTU7	1.52	1.48	FJ543133.1	Clone OP410 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	97%
OTU23	1.26	1.45	FJ543132.1	Clone OP444 (Acidobacteria; Holophagae; TK85)	99%
OTU54	1.11	1.14	FJ269280.1	Clone XA2F04F (Acidobacteria; Holophagae; TK85)	98%
OTU30	1.05	0.78	GU460749.1	Clone ST19_5m_clone18 (Actinobacteria; Actinobacteria; PeM15)	99%
OTU21	0.56	0.91	JN655343.1	Clone PO10-3-1_C24 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	97%
OTU32	0.73	0.75	JN874203.1	Reset_28F03 (Planctomycetes; Planctomycetacia; Planctomycetales; Planctomycetaceae)	98%
OTU10	0.71	0.66	FJ543132.1	Clone OP444 (Acidobacteria; Holophagae; TK85)	99%
OTU31	0.58	0.62	JN210639.1	Clone T534deg9 (Acidobacteria; Holophagae; TK85)	98%
OTU17	0.86	0.39	FJ543133.1	Clone OP410 (Chloroflexi; Caldilineae; Caldilineales; Caldilineaceae; *Caldilinea*)	96%
OTU20	0.57	0.61	JN210639.1	Clone T534deg9 (Acidobacteria; Holophagae; TK85)	98%

S. aureus, *E. coli*, *V. parahaemolyticus*, *P. aeruginosa*, and *C. albicans* to the 51 sponge isolates was 84.31, 23.53, 47.06, 7.84, and 39.22% (Fig. 2b), respectively, and the percentage of susceptibility to the 51 sponge isolates with inhibition zone diameters > 5 mm was 56.86, 11.76, 27.45, 3.92, and 25.49%, respectively.

Cytotoxic activity analysis

Twenty-seven isolates (including 13 bacteria and 14 actinomycetes) for which an inhibition zone > 10 mm in diameter was observed against at least one test strain, or that were able to inhibit at least four test strains in secondary screening, were selected for evaluation of cytotoxic activity. Among these isolates, 29.6% (eight isolates, AIAC10-11, AIAC1-21, GIC10-1, GIC1-13, M1C1-37, PYAC20-24, PYAU1-1, PYAN10-21), including one bacterium and seven actinomycetes, were found to exert cytotoxic activity against at least five of the seven human cancer cell lines (Table 4). We consider that these eight isolates have potential for further study, in particular actinomycetes AIAC10-11 and GIC1-13, and bacterium PYAN10-21, which had an $IC_{50} < 1$ μg/ml for at least two cancer cell lines.

Identification of bacteria producing antibiotics

16S rRNA gene sequences of the 51 selected isolates that exhibited antimicrobial activities were sequenced and subsequently aligned to construct phylogenetic trees (Fig. 3a, b). It should be noted that these 51 isolates were, therefore, neither a representative sample of culturable bacteria nor a representative sample of culturable bacteria with antimicrobial activities. Among the 51 isolates, 9 belonged to the phylum Firmicutes, 32 to the phylum Actinobacteria, and 10 to the phylum Proteobacteria. In addition, we used the Ribosomal Data Project (RDP) classifier tool (Cole et al. 2005) to classify bacteria based on their 16S rRNA sequences. The 51 isolates were classified into six genera: *Streptomyces* (30 isolates), *Pseudoalteromonas* (10), *Bacillus* (8), *Brevibacterium* (1), *Micromonospora* (1), and *Paenibacillus* (1). *Streptomyces*, *Pseudoalteromonas*, and *Bacillus* were the three major genera, accounting for 94% of the antibiotic-producing isolates.

Overlap of antimicrobial isolates and 454 sequences

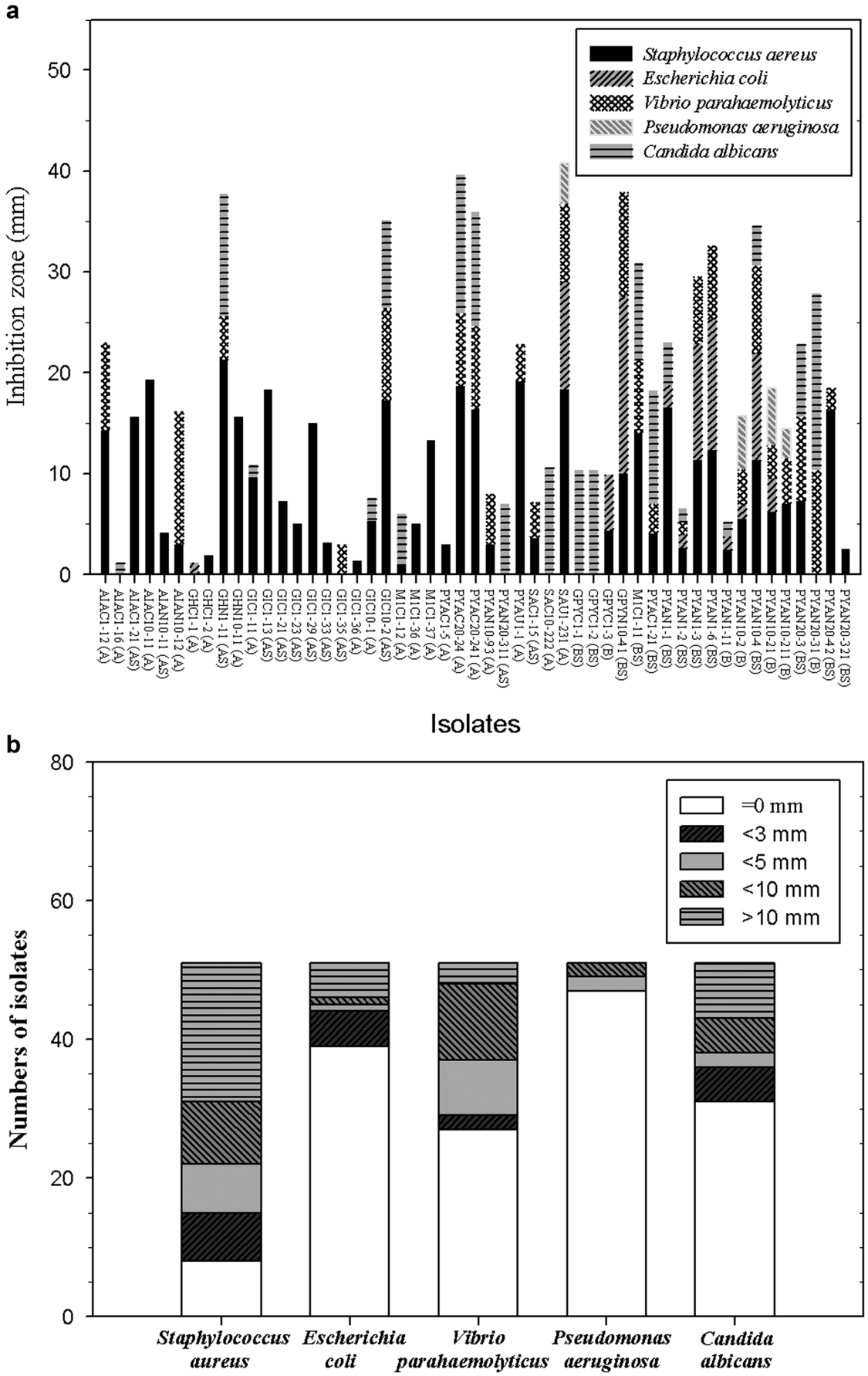
In the present study, we found that all of the antimicrobial isolates obtained from the sponge samples belonged to three bacterial phyla, namely Actinobacteria (62.8%), Proteobacteria (19.6%), and Firmicutes (17.4%), among which 70 (8.56% of the total OTUs), 187 (8.56%), and 29 OTUs (3.55%), respectively, were found in the 454 pyrosequencing data set (Supplementary File 1). At the genus level, only three genera, *Pseudoalteromonas*, *Streptomyces*, and *Bacillus*, were found to each have one OTU, and no *Brevibacterium*, *Micromonospora*, or *Paenibacillus* OTUs were found in the pyrosequencing data set (Supplementary File 1). BLAST comparison of the 16S rRNA gene sequences of the antimicrobial isolated bacteria in the pyrosequencing data indicated that only OTU692 and OTU618, found within the *T. swinhoei* metagenome, were closely related (98 and 96% identities, respectively) to 16S rRNA genes from the eight *Pseudoalteromonas* and one *Streptomyces* isolates, respectively. This lack of overlap between culture-based isolates and metagenomic sequences was consistent with studies of the microbiomes of sponges *Rhopaloeides odorabile* (Webster and Hill 2001) and *Haliclona simulans* (Kennedy et al. 2009).

Discussion

Many researchers believe that symbiotic microbes of marine invertebrates are an untapped source of bioactive compounds (Burgess et al. 1999; Nithyanand et al. 2011). However, just a small fraction of these compounds has been identified and studied. In the present study, bacteria associated with the marine sponge *T. swinhoei* obtained from southern Taiwan were systematically studied and screened for production of antibiotics. Of a total of 700 cultured isolates, including 606 bacteria and 94 actinomycetes, 105 strains (15%) showed antibiotic activity towards at least one indicator microbe used in primary screening. A significantly higher percentage of antibiotic-producing isolates was detected in the actinomycetal isolates (42.55%) than in the bacterial isolates (10.73%). The fraction (12%) of antimicrobial-producing bacteria in our results was consistent with several previous studies; for example,

Table 3 Number of strains isolated, number (percentage) of isolates exhibiting antimicrobial activity, and number of antimicrobial isolates with seawater requirements from marine sponge *Theonella swinhoei* using the agar block method in primary screening

Group	Number of isolates	Number of antimicrobial isolates	Number of anti-*Staphylococcus aureus*	Number of anti-*Vibrio parahaemolyticus*	Number of anti-*Candida albicans*	Number of antimicrobial isolates with seawater requirement
Bacterium	606	65 (10.73%)	38 (6.27%)	15 (2.48%)	25 (4.13%)	14 (21.54%)
Actinomycete	94	40 (42.55%)	33 (35.11%)	8 (8.51%)	12 (12.77%)	12 (30.00%)
Total	700	105 (15.00%)	71 (10.14%)	21 (3.29%)	37 (5.29%)	26 (24.76%)


a
Staphylococcus aereus
Escherichia coli
Vibrio parahaemolyticus
Pseudomonas aeruginosa
Candida albicans
Inhibition zone (mm)
0
10
20
30
40
50
ALAC1-12 (A)
ALAC1-16 (A)
ALAC1-21 (AS)
ALAC10-11 (A)
ALAN10-11 (AS)
ALAN10-12 (A)
GHC1-1 (A)
GHC1-2 (A)
GHN1-11 (AS)
GHN10-11 (A)
GIC1-11 (A)
GIC1-13 (AS)
GIC1-21 (AS)
GIC1-23 (AS)
GIC1-29 (AS)
GIC1-33 (AS)
GIC1-35 (AS)
GIC1-36 (A)
GIC10-1 (A)
GIC10-2 (AS)
M1C1-12 (A)
M1C1-36 (A)
M1C1-37 (A)
PYAC1-5 (A)
PYAC20-24 (A)
PYAC20-241 (A)
PYAN10-93 (A)
PYAN20-311 (AS)
PYAU1-1 (A)
SAC1-15 (AS)
SAC10-222 (A)
SAU1-231 (A)
GPYC1-1 (BS)
GPYC1-2 (BS)
GPYC1-3 (B)
GPYN10-41 (BS)
M1C1-11 (BS)
PYAC1-21 (BS)
PYAN1-1 (BS)
PYAN1-2 (BS)
PYAN1-3 (BS)
PYAN1-6 (BS)
PYAN1-11 (B)
PYAN10-2 (B)
PYAN10-4 (BS)
PYAN10-21 (B)
PYAN10-211 (B)
PYAN20-3 (BS)
PYAN20-31 (B)
PYAN20-42 (BS)
PYAN20-321 (BS)
Isolates
b
=0 mm
<3 mm
<5 mm
<10 mm
>10 mm
Numbers of isolates
0
20
40
60
80
Staphylococcus aureus
Escherichia coli
Vibrio parahaemolyticus
Pseudomonas aeruginosa
Candida albicans

◀ **Fig. 2** **a** Distribution of the diameter of inhibition zone size; **b** distribution of the diameter of inhibition zone size against different indicator strains for strains isolated from marine sponge *Theonella swinhoei* using the agar block method in secondary screening. Letters in parentheses after the strain indicate (a) actinomycetes and (b) bacteria

Hentschel et al. (2001) studied the antimicrobial activity of the sponge *Aplysina*, and found that 11.35% of the bacterial isolates possessed antimicrobial activity; and Zhang et al. (2009) showed that 18% of the bacterial isolates obtained from four sponge species were antibiotic producers. In general, the percentage of microbial isolates with antimicrobial activity in sponges varies between 10 and 50% (Flemer et al. 2012; Hentschel et al. 2001; Kennedy et al. 2009; Santos et al. 2010). It should be noted that the percentage can be higher if the number of test strains is increased and more diverse conditions are used for the assay (Kennedy et al. 2009).

Phylogenetic analysis (Fig. 3a, b) showed that *Streptomyces* and *Pseudoalteromonas* were the two major genera that contained 78.43% of the antibiotic producers in this study. This result was consistent with the study by Kennedy et al. (2009), which showed that most of the isolates from Demospongiae sponge *Haliclona simulans* that exhibited antimicrobial activity belonged to the genera *Streptomyces* (36.5%) and *Pseudoalteromonas* (23.1%). In this study, most of the *Pseudoalteromonas* isolates obtained from *T. swinhoei* inhibited at least one Gram-positive and one Gram-negative indicator bacteria, whereas only three of the nine tested *Pseudoalteromonas* isolates showed antifungal activities against *C. albicans*. *Pseudoalteromonas* contains several species that are known to produce biologically active compounds with antimicrobial and antifouling effects (Wilson et al. 2010). They are often found in symbiosis with marine eukaryotic hosts, such as sponges, coral, and tunicates (Hentschel et al. 2001). In previous culture-based studies, Hentschel et al. (2001) and Kennedy et al. (2009) isolated several *Pseudoalteromonas* species from Irish and Mediterranean sponges, respectively, and found that they exhibited no antimicrobial activity against either *E. coli* or *S. aureus*, and only antimicrobial activity against a Gram-negative marine bacterium, respectively, which was significantly less activity than that demonstrated by our results. These results might indicate that different isolates from different sponges might adapt to dissimilar host environments and produce varying antibiotics, suggesting the need to isolate bacteria from different marine sponges.

Overall, the bacterial assemblage of sponge *T. swinhoei* was composed mainly of Acidobacteria, Chloroflexi, Planctomycetes, Proteobacteria, Actinobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, Verrucomicrobia, and Deferribacteres, as well as candidate phylum TM6 and TM7 (Fig. 1). This high level of microbial richness at the phylum level was consistent with several previous culture-independent surveys of microbial communities of Demospongiae sponges, which are known to host a diverse range of bacteria. The bacterial communities associated with *T. swinhoei* were recently described for samples obtained from Palau, Israel, and Japan (Hentschel et al. 2002). Comparison of the bacterial flora of these samples with those identified in this study at the phylum level showed that some of the major phyla, such as Acidobacteria, Chloroflexi, Proteobacteria, Actinobacteria, and Cyanobacteria, were maintained, while Planctomycetes and Firmicutes were not, suggesting that the sponge host might have the ability to establish new bacterial flora to adapt to different environments.

OTU1, the most abundant 16S rRNA OTU of the sponge *T. swinhoei*, which represented almost half (approximately 47%) of the total sequence (Table 2), showed the best hit with a sequence (FJ543132, 98% similarity) obtained from sponge *Acanthostrongylophora* sp., belonging to the phylum Acidobacteria and class Holophagae. In addition, 11 of the 20 most abundant OTUs also belonged to Acidobacteria,

Table 4 IC_{50} values of crude extracts of bacterial strains isolated from marine sponge *Theonella swinhoei* against cancer cell lines MCF-7, MDA-MB-231, and T47-D from breast tumors, MOLT-4 and K-562 from leukemia, and DLD-1 and HCT-116 from colorectal cancer using the MTT method

Isolate	IC_{50} (μg/ml)						
	K-562	Molt 4	DLD-1	T47-D	HCT-116	MCF-7	MDA-MB-231
AIAC10-11 (A)	< 1	< 1	4.61	23.58	–	8.95	31.91
AIAC1-21 (A)	< 25	< 25	–	3.08	9.96	–	1.02
GIC10-1 (A)	< 25	< 25	11.81	3.51	21.19	–	–
GIC1-13 (A)	< 1	< 1	24.73	18.7	–	–	3.57
M1C1-37 (A)	NA	0.26	4.61	3.95	2.87	13.2	2.89
PYAC20-24 (A)	< 25	< 25	1.13	23.58	–	8.95	31.91
PYAU1-1 (A)	< 15.625	< 15.625	13.68	2.42	27.64	15.96	3.65
PYAN10-21 (B)	16.04	0.67	0.05	0.57	12.19	0.52	0.88

– no activity

NT no test

representing 71.54% of the total sequence associated with *T. swinhoei*. This group of bacteria was also the most abundant bacterial phylum in Palauan and Israeli *T. swinhoei* (Hentschel et al. 2002), both representing about 40% of the total sequence; this was very different from the 71.54% observed in the present study, suggesting that geographic location or environmental factors might play roles in sponge-associated microbial communities, rather than species-specific factors. O'Connor-Sanchez et al. (2014) reported that a single Acidobacteria OTU dominated the bacterial community of Demospongiae sponge *Hyrtios* sp. collected in the Mexican Caribbean, with a relative abundance of 80%, which was consistent with our results.

Using the 16S rDNA-based molecular method, bacteria in the phylum Acidobacteria have been found to be highly diverse, and are consistently detected in different habitats, including soil, sediment, freshwater, marine, and wastewater environments (Barns et al. 2007; Ward et al. 2009), which suggests that they play an important ecological role and have varied functions. They are particularly abundant in soil and sediment habitats, comprising 10 to 50% of the total bacterial abundance (Barns et al. 2007). Previous studies of microbes associated with marine invertebrates have also revealed that Acidobacteria can inhibit several sponge (Hardoim and Costa 2014; O'Connor-Sanchez et al. 2014; Schmitt et al. 2012; Simister et al. 2013) and coral species (Closek et al. 2014; Lin et al. 2016; Ng et al. 2015). Currently, our knowledge of the ecological functions served by this group of bacteria is still scarce (O'Connor-Sanchez et al. 2014). The high abundance of Acidobacteria in the present study might be representative of adaption to the environmental conditions at Nanwan Bay in southern Taiwan. In addition, the Acidobacteria symbiont might produce secondary metabolites to inhibit the growth of other microbes (O'Connor-Sanchez et al. 2014). However, we did not isolate any Acidobacteria with antimicrobial activity from *T. swinhoei*, probably because

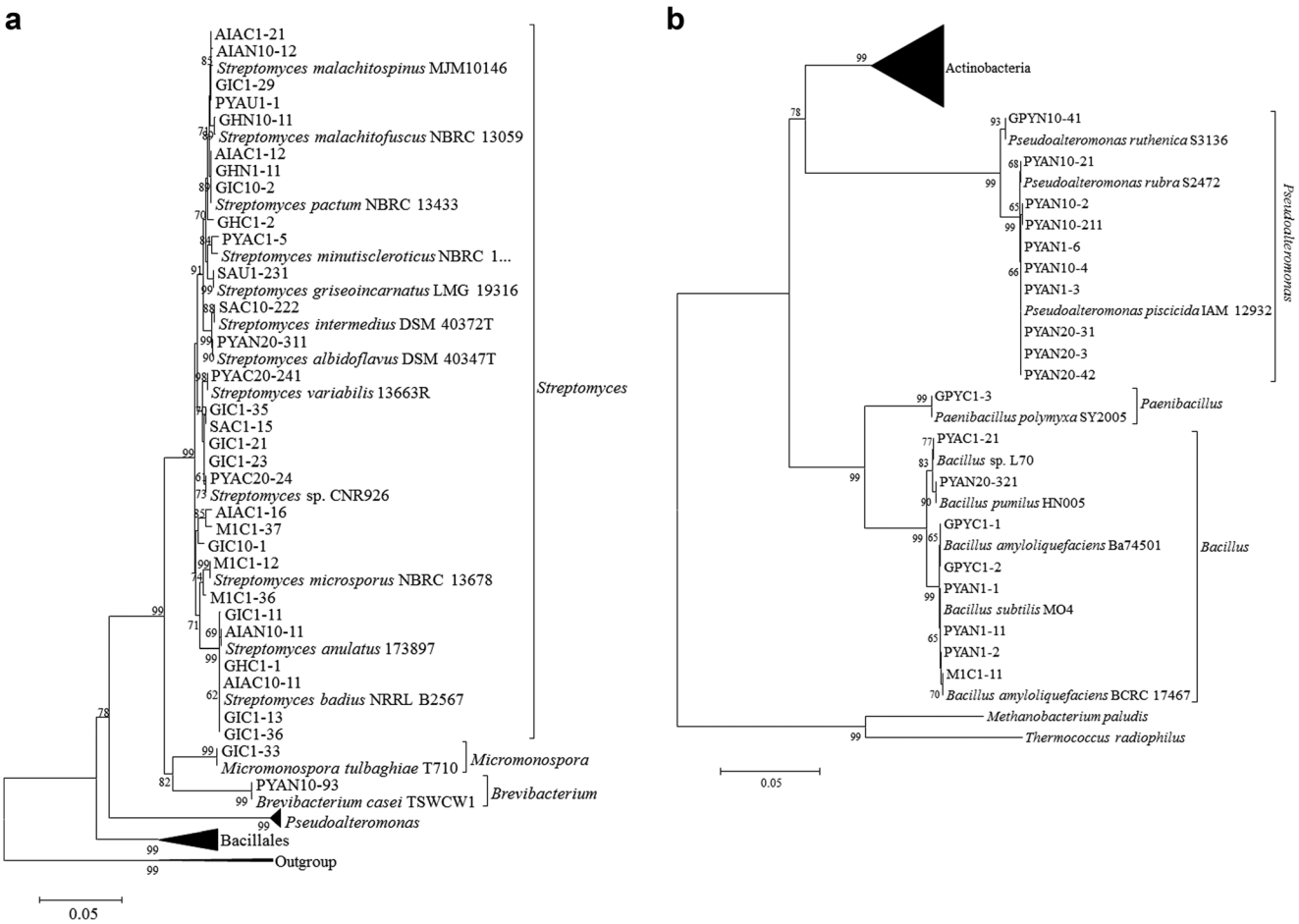


Fig. 3 Phylogenetic trees of selected bacterial strains isolated from sponge *Theonella swinhoei.* The trees were constructed by comparison of an approximate 650-bp region of the 16S rRNA gene sequence using neighbor-joining analysis of a distance matrix with Kimura's two-parameter model. Bootstrap values (expressed as percentages of 100 replications) greater than 60% are shown at branch points. The 16S rRNA sequences of *Methanobacterium paludis* and *Thermococcus radiophilus* were used as outgroups. The scale bar represents 0.05 substitutions per nucleotide position

Acidobacteria are very difficult to cultivate and slow-growing, suggesting that different culturing strategies may needed to culture Acidobacteria.

We observed that Planctomycetaceae, a member of the Planctomycetes family, was the second most abundant family identified in *T. swinhoei*, with a relative read abundance of 10.67% (in identified families) (Supplementary File 1). Planctomycetes have been found in a variety of marine, freshwater, and terrestrial environments (Pimentel-Elardo et al. 2003), and have also been found to be associated with marine eukaryotes (Kellogg et al. 2016; Mohamed et al. 2010). Recently, several members of this family were found to mediate anaerobic ammonia oxidation (anammox), which is an important biological nitrogen removal process that has been postulated to generate 50% of the nitrogen in the atmosphere (Jetten 2008). Although the diversity of Planctomycetaceae (including 178 OTUs or five genera) was high in *T. swinhoei*, only 13 OTUs (22 reads), which were all identified as OM190 (Silva taxonomy), were found to be distantly related to anammox bacteria (Ye et al. 2016). The OM190 lineage has been found to be associated with kelp *Laminaria hyperborea* (Bengtsson and Ovreas 2010) and marine sponge *Mycale laxissima* (Mohamed et al. 2010). The presence of anammox OTUs might be important for the nitrogen cycle in *T. swinhoei*, and clearly warrants further study.

Phylum Chloroflexi (green non-sulfur bacteria) was the second most abundant phylum (19.6% of total reads) in this study. A total 142 Chloroflexi OTUs, which accounts for 17.36% of all OTUs, was identified, suggesting the diversity of Chloroflexi community in *T. swinhoei* was also high. This group of bacteria was also found to be a dominant bacterial phylum in several marine sponges, such as *Rhopaloeides odorabile* (39–41% of total reads) (Simister et al. 2012) and *Ecionemia alata* (19–28%) (Cardenas et al. 2014). Recently, Schmitt et al. (2011) compared the Chloroflexi community structure between high microbial abundance (HMA) and low microbial abundance (LMA) sponges and found that Chloroflexi bacteria are more diverse and abundant in HMA than in LMA sponges, which is consistent with our results for *T. swinhoei* (also HMA). At present, our knowledge regarding Chloroflexi in sponges is still largely based on the application of cultivation-independent methods (Schmitt et al. 2011). Further clarifying the role of sponge-associated Chloroflexi and determining its ecological significance remains a big challenge for future research.

The eight most dominant Chloroflexi OTUs identified were OTU3, OTU5, OTU6, OTU4, OTU9, OTU7, OTU21, and OTU17, comprising 12.07% of total reads (Table 2). Although two Chloroflexi classes, Caldilineae and Anaerolineae, were identified in this study, all the eight Chloroflexi OTUs belong to genius *Caldilinea* (class Caldilineae). Caldilineae is a non-photosynthetic and ubiquitous class with 16S rRNA gene sequences reported in soils (Breuker et al. 2011), marine sediments (Schmitt et al. 2011), activated sludge (Kindaichi et al. 2012), and marine animals, such as sponge (Schmitt et al. 2011). BLAST results showed these eight OTUs were all closely related to bacterial sequences in marine sponges. Interesting, similar to Planctomycetes OTUs, members of this class were also found to exist in anammox reactor (Kindaichi et al. 2012), suggested that anammox reaction could be an important pathway for ammonium removal in host. To the author's knowledge, there has been no published study of antibiotics producing Chloroflexi. However, several PKS genes were identified in a Chloroflexi genome (Kiss et al. 2011), suggesting the possibility of using Chloroflexi as antibiotics producer.

In the present study, eight isolates of *Pseudoalteromonas* and one isolate of *Streptomycetes* were found to be shared between the cultured isolates and 454 sequences. This discrepancy between the major groups of culture-based isolates and deep pyrosequencing sequences was in agreement with the results of several previous studies (Kennedy et al. 2009; Montalvo et al. 2014). The inconsistency is possibly due to the percentage of bacteria isolated was a very small part of the bacterial community of *T. swinhoei*, and therefore cannot detect in pyrosequencing sequences (Kennedy et al. 2009). Our study highlights that the culture-based method can address some of the limitations of NGS sequencing, and is worthwhile not only in order to isolate bacteria for biotechnology usage, but also to increase the detection of bacterial community diversity in sponges.

Eight isolates (Table 4) that exhibited strong or wide-spectrum antibiotic activities and cytotoxic activity against cancer cells provided further evidence that culturable sponge microflora is an important source of biologically active compounds. For isolate GIC10-1, the 16S rRNA gene BLAST results showed a 100% identity with *Streptomycetes* sp. OPMA00072 (Genbank accession no. AB896819). The crude extract of this isolate possessed antimicrobial activity against two indicator microbes and cytotoxic activity against three cancer cells, which suggested that it is a good candidate for further natural product isolation and characterization. In fact, a new compound, bafilomycin M, was recently isolated from the culture broth of GIC10-1 (Chen et al. 2016). Thus, our study demonstrated that microbes associated with the marine sponge *T. swinhoei* can be a source of natural antimicrobial agents, and these antibiotic-producing bacteria may play an ecologically important role in the relationship between microbial symbiont and sponge host.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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