

## Isolation of marine bacteria with antimicrobial activities from cultured and field-collected soft corals

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**Abstract** Bacteria associated with eight field-collected and five cultured soft corals of *Briareum* sp., *Sinularia* sp., *Sarcophyton* sp., *Nephtheidae* sp., and *Lobophytum* sp. were screened for their abilities in producing antimicrobial metabolites. Field-collected coral samples were collected from Nanwan Bay in southern Taiwan. Cultured corals were collected from the cultivating tank at National Museum of Marine Biology and Aquarium. A total of 1,526 and 1,138 culturable, heterotrophic bacteria were isolated from wild and cultured corals, respectively; seawater requirement and antimicrobial activity were then assessed. There is no significant difference between the ratio of seawater-requiring bacteria on the wild and cultured corals. The ratio of antibiotic-producing bacteria

within the seawater-requiring bacteria did not differ between the corals. Nineteen bacterial strains that showed high antimicrobial activity were selected for 16S rDNA sequencing. Three strains could be assigned at the family level (Rhodobacteraceae). The remaining 16 strains belong to eight genera: *Marinobacterium* (2 strains), *Pseudoalteromonas* (1), *Vibrio* (5), *Enterovibrio* (1), *Tateyamaria* (1), *Labrenzia* (2), and *Pseudovibrio* (4). The crude extract from bacteria strains CGH2XX was found to have high cytotoxicity against the cancer cell line HL-60 ( $IC_{50} = 0.94 \mu\text{g/ml}$ ) and CCRF-CEM ( $IC_{50} = 1.19 \mu\text{g/ml}$ ). Our results demonstrate that the marine bacteria from corals have great potential in the discovery of useful medical molecules.

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### Introduction

An increase of bacterial resistance to existing antibiotics has led to the search for new drugs, especially antibiotics. Bioactive compounds are traditionally screened from terrestrial microorganisms. However, the opportunity of finding novel antibiotics from terrestrial microorganisms has diminished (Hong et al. 2009a). The environments between oceanic and terrestrial systems are very different. As such, marine microorganisms may produce bioactive compounds not found in terrestrial habitats (Penesyan et al. 2009). It has been reported that marine invertebrates harbor a higher population of bacteria able to produce novel bioactive compounds (Burgess et al. 1999; Nithyanand et al. 2011). Previous studies on microbes associated with sponges have revealed

that some bacteria have a great potential for producing useful natural products (Li 2009). However, coral-associated microbes and their produced metabolites have received little attention (Li 2009). Thus, coral reef ecosystems could be a largely unexplored source for marine microorganisms producing bioactive compounds.

Like sponges, many microbes, including bacteria (Rohwer et al. 2002; Chiou et al. 2010), fungi (Bentis et al. 2000; Wegley et al. 2007), and archaea (Kellogg 2004; Ferris et al. 1996), live in the skeleton, tissues, and surface mucus layer of corals. Some of the associations between corals and microbes are stable as well as species-specific and often distinct from the surrounding planktonic microorganisms (Rohwer et al. 2001; Rohwer et al. 2002). The functions of these coral-associated microorganisms are mostly unknown. Yet, it is suspected that they could play an important role in coral health, nutrition, and disease (Hong et al. 2009b).

Recently, several attempts have been made to culture and assay coral-associated isolates for the production of antimicrobial substances (Nissimov et al. 2009; Shnit-Orland and Kushmaro 2009; Radjasa et al. 2008; Lampert et al. 2006; Ritchie 2006). Nissimov et al. (2009) surveyed the antimicrobial activities of bacterial isolates from the mucus of coral *Oculina patagonica* and found that several bacterial isolates displayed antagonistic activities toward coral pathogens *Vibrio shilonii*, *V. coralliilyticus*, and *Serratia marcescens*. Another study has shown that corals also harbor diverse species of actinomycetes (Lampert et al. 2008; Lampert et al. 2006; Lombo et al. 2006) and some of these actinomycetes can inhibit the biofilm formation of *Streptococcus pyogenes* (Nithyanand et al. 2010). Reshef et al. (2006) suggested that these antibiotic-

producing bacteria may play an important role in the first-line defense for coral against invading pathogens.

In this study, field-collected and cultured soft corals were used as a source for screening marine bacteria that exhibit antimicrobial activity using a culture-based method. The antimicrobial potentials of these bacteria were assessed and the difference of bacterial flora with antimicrobial activities between field-collected and cultured coral was compared. We hypothesized that the bacterial flora with antimicrobial activities would differ between the field-collected and cultured soft corals. The original objective of this study was primarily to isolate marine actinomycetes. However, only few actinomycetes were isolated and none of them was seawater-dependent. We, therefore, decided to study the antimicrobial activity of marine bacteria.

## Materials and methods

### Collection of coral samples

Thirteen coral samples, including five cultured corals and eight field-collected corals, were collected in this research (Table 1). The cultured soft corals were collected in January and March 2009 from an 80 ton cultivating tank located in the National Museum of Marine Biology and Aquarium (NMMBA) at 33 ‰ salinity, 25–26 °C. They included four soft coral genera (*Briareum* sp., *Lobophytum* sp., *Nephtheidae* sp., and *Sarcophyton* sp.). The field-collected soft corals were obtained in January, April, October, and November 2009 from the coast of Nanwan Bay (21°57'10.69"N, 120°46'8.29"E), Kenting National Park, southern Taiwan, at depths of about 10 m. The

**Table 1** The results of soft coral sample collection as well as the number of selective and seawater-requiring bacterial strains isolated from soft coral

Sample	Genus of coral	Origin of coral	Health status	Sampling date	Number of isolate	Number of seawater-requiring isolate (%)
A	<i>Briareum</i> sp.	Cultured	Healthy	Mar, 2009	515	366 (71.07)
B	<i>Briareum</i> sp.	Cultured	Diseased	Jan, 2009	359	120 (33.43)
C	<i>Lobophytum</i> sp.	Cultured	Healthy	Mar, 2009	158	111 (70.25)
D	<i>Nephtheidae</i> sp.	Cultured	Healthy	Mar, 2009	64	53 (82.81)
E	<i>Sarcophyton</i> sp.	Cultured	Healthy	Mar, 2009	42	31 (73.81)
F	<i>Briareum</i> sp.	Field-collected	Healthy	Nov, 2009	816	790 (96.81)
G	<i>Nephtheidae</i> sp.	Field-collected	Healthy	Nov, 2009	77	69 (89.61)
H	<i>Sarcophyton</i> sp.	Field-collected	Healthy	Apr, 2009	96	68 (70.83)
I	<i>Sarcophyton</i> sp.	Field-collected	Healthy	Apr, 2009	84	59 (70.24)
J	<i>Sarcophyton</i> sp.	Field-collected	Healthy	Nov, 2009	41	26 (63.41)
K	<i>Sarcophyton</i> sp.	Field-collected	Healthy	Nov, 2009	152	136 (89.47)
L	<i>Simularia</i> sp.	Field-collected	Healthy	Jan, 2009	51	32 (62.75)
M	<i>Simularia</i> sp.	Field-collected	Healthy	Oct, 2009	209	168 (80.38)
	Total				2,664	2,030 (76.2)

field-collected corals were composed of four soft coral genera (*Simularia* sp., *Sarcophyton* sp., *Briareum* sp., and *Nephtheidae* sp.). All corals appeared to be healthy at the time of sampling except a cultured *Briareum* sp. The field-collected and cultured coral samples were kept at 4 °C after collection, transported to the laboratory, and processed within 4 h.

#### Culture of coral-associated bacteria

Approximately 2 g of soft coral was thoroughly washed twice with filtered (0.22 µm) seawater, ground with a sterilized mortar and pestle, and transferred to a 50-ml centrifuge tube. The soft coral was then vigorously vortexed for 3 min after adding 10 ml filtered seawater. The sample was serially diluted with seawater and spread on three different actinomycete-selective media: the M1 agar (M1): 10 g/l starch, 4 g/l yeast extract, 2 g/l peptone, and 15 g/l agar (Jensen et al. 2005); the Gause mineral agar (GH): 20 g/l starch, 1 g/l KNO<sub>2</sub>, 0.5 g/l K<sub>2</sub>HPO<sub>4</sub>, 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.05 g/l nystatin, 0.05 g/l cycloheximide, and 15 g/l agar (Ivanitskaia et al. 1978); and the glycerol asparagine agar (GA): 12.5 g/l glycerol, 1 g/l asparagine, 1 g/l K<sub>2</sub>HPO<sub>4</sub>, 1 g/l NaCl, 0.5 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g/l FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.001 g/l CuSO<sub>4</sub>, 0.001 g/l MnSO<sub>4</sub>, 0.001 g/l ZnSO<sub>4</sub>, 0.05 g/l nystatin, 0.05 g/l cycloheximide, and 15 g/l agar (Dharmaraj and Sumantha 2009). All the above media were supplemented with 80 % of sea water. Plates were incubated at 25 °C for 5 days. Individual colonies were picked and streaked onto fresh M1 agar 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 solutions.

#### Detection of seawater requirement

All the isolated strains were streaked on M1 agar supplemented with 0 and 80 % (v/v) of seawater. After streaking the plates with test strains, the plates were incubated at 25 °C for 7 days. Isolates that only grow in M1 agar with 80 % seawater were designated as obligate marine bacteria and all the following biological activity screenings were conducted using these obligate marine bacteria.

#### Indicator microorganisms

Bacterial strains *Vibrio parahaemolyticus* (BCRC 10806) and *Pseudomonas aeruginosa* (BCRC 10303), as well as the fungal strain *Candida albicans* (BCRC 22903), were obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). Bacterial strain *Vibrio shilonii* (ATCC BAA-91) was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA).

Bacterial strains *Staphylococcus aureus* (ATCC 12600) and *Escherichia coli* (ATCC 11775) were provided by Dr. J. K. Liu, Department of Biological Sciences, National Sun Yat-sen University, Taiwan.

Indicator microbes were cultured as follows: *V. parahaemolyticus*, *P. aeruginosa* and *V. shilonii* were cultured in marine broth at 25 °C for 24 h and maintained on the marine agar. *C. albicans* was cultured in YM broth at 30 °C for 48 h and maintained on the YM agar. *S. aureus* and *E. coli* was cultured in LB broth at 37 °C for 18 h and maintained on the LB agar.

#### Determination of antimicrobial activity

The screening method consisted of two steps: primary and secondary screening. The primary screening was used to select the antimicrobial isolates against a Gram-negative strain (*Vibrio shilonii*), a Gram-positive strain (*Staphylococcus aureus*), and a eukaryotic fungus (*Candida albicans*). Isolates that exhibited an inhibition zone >1 mm in diameter against at least one test strain in the primary screening were selected for secondary screening against six pathogens *E. coli*, *V. parahaemolyticus*, *P. aeruginosa*, *V. shilonii*, *S. aureus*, and *C. albicans*.

An agar block method (Stern et al. 2006) was used for the primary and secondary screening: each coral isolate was spread evenly on the surface of M1 agar plate, and incubated for 3–5 days at 25 °C. Cylindrical agar blocks (8-mm diameter) were excised from well-growth agar. The agar blocks were placed directly on the top of a new agar plate freshly spread with a fixed amount of indicator microorganisms (10<sup>8</sup> cells/ml). The agar plate containing the agar blocks and newly inoculated indicator microorganism was incubated for 24 h at the proper temperature for the indicator microorganism. The antibacterial activity was measured in terms of diameter of the inhibition zone with triplication.

#### Crude extract preparation

Marine isolates were cultured in 500-ml flasks containing 100 ml M1 medium with 80 % 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

Cell lines CCRF-CEM (human T lymphoblastic leukemia), HL-60 (human promyelocytic leukemia) and normal

PBMC (normal peripheral blood mononuclear cells) were purchased from the ATCC. 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<sub>2</sub>.

The cytotoxic activity of the crude extract was assessed by a colorimetric method using 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetra-zolium bromide (MTT; Lu et al. 2009).

#### Bacterial growth in different media

About 2 g of cultured *Briareum* sp. was washed and crushed as mentioned before. The coral sample was then serially diluted with seawater and spread on four different media: marine agar, M1, GH, and GA. After incubation for 5 days at 25 °C, the bacterial colonies that developed on the plates were counted and numbers were expressed in colony-forming unit (CFU).

#### 16S rRNA gene sequencing

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

#### Phylogenetic analysis

The nucleotide sequences of the 16S rRNA gene fragments were first compared with those in GenBank databases using the basic local alignment search tool (BLAST; Altschul et al. 1990) algorithm to identify the closest bacterial neighbors. To build the phylogenetic tree, sequences were aligned using the multiple sequence alignment program from MEGA 4 program (Tamura et al. 2007) using the ClustalW method (Thompson et al. 1994). A neighbor-joining tree (Saitou and Nei 1987) was constructed with Kimura's two-parameter model (Kimura 1980) using the MEGA 4 program. The statistical significance of tree branches was evaluated by bootstrap analysis involving the construction of 1,000 trees from randomly resampled data.

#### Statistics

Student's *t* tests and Mann–Whitney U tests were performed with the software SPSS version 12.0. In order to make the data set a better approximation of normal distribution, the percentage of bacterial isolates with seawater

requirement and antimicrobial activity was converted into an arcsin value before performing the Student's *t* test and Mann–Whitney U test.

All results from biological activity assays were expressed as mean ± standard error.

#### Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study have been deposited in the GenBank database under the accession numbers from JQ342679 to JQ342697.

## Results

#### Sample collection, bacteria isolation and seawater requirement assay

It is important to ensure that the isolates were truly coral-associated microbes and are not due to contamination from the land. We estimated the degree of terrestrial microorganism contamination in the marine corals by calculating the percentage of seawater tolerated isolated out of the total isolated. We assumed that a low percentage of seawater-sensitive bacteria in a sample could be indicative of low contamination from terrestrial bacteria.

Using three different culture media, a total of 2,664 heterotrophic bacteria were isolated from thirteen coral samples and of these 1,138 and 1,526 bacterial strains were from cultured (sample A-E) and field-collected (sample F-M) coral samples, respectively (Table 1).

The number and percentage of isolates which showed seawater requirement for each species of coral are also presented in Table 1. Among all the isolated strains (2,664), 76.2 % (2,030) isolates had a seawater requirement. Isolates from field-collected *Briareum* sp. (sample F) had the highest percentage of seawater-requiring bacteria (96.81 %), while isolates from diseased cultured *Briareum* sp. (sample B.) had the lowest percentage of seawater-requiring bacteria (33.43 %). Among isolates from field-collected corals, isolates from *Sinularia* sp. (sample L) had the lowest percentage of seawater-requiring bacteria (62.75 %), while isolates from *Nephtheidae* sp. (sample D) had the highest percentage of seawater-requiring bacteria (82.81 %) among isolates from cultured corals.

The mean values and standard errors of the percentage of seawater-requiring isolates for each coral sample observed from cultured and field-collected coral sample were 66.27 ± 8.51 % and 77.94 ± 4.60 %, respectively. There were no differences in the percentage of seawater-requiring bacteria between cultured (n = 4, excluding sample B) and field-collected coral isolates (Student's *t* test; *P* > 0.05). However, the total average of the





**Fig. 1** Agar block assay for bacterial strains isolated from soft corals against indicator bacterium (2XX indicates isolate CGH2XX)

percentage of seawater-requiring isolates for field-collected corals (88.47 %) was higher than for cultured corals (72.02 %).

#### Antimicrobial activity assay

All isolates that showed a seawater requirement were subsequently screened for their antimicrobial activity. The antimicrobial test was carried out in primary screening by

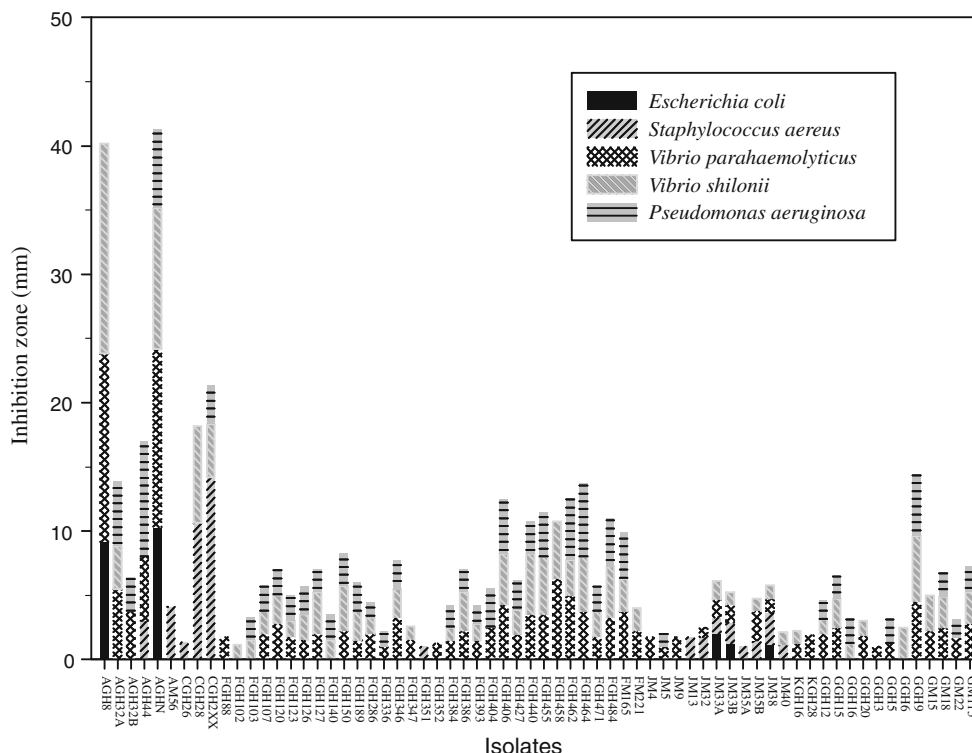
agar block methods against three pathogens *Vibrio shilonii*, *Staphylococcus aureus*, and *Candida albicans* (Fig. 1). For each coral sample, the percentage of isolates displayed antimicrobial activity and the percentage of isolates against the three indicator bacteria are shown in Table 2. Among all the tested isolates (2,030), 6.55 % (133) of the isolates showed antimicrobial activity against one or more of the indicator microorganisms. 4.68 % (95) strains showed activity against *V. shilonii*, 3.82 % (78) against *S. aureus*, and 1.97 % (40) against both *V. shilonii* as well as *S. aureus*. No antimicrobial activity against fungal strain *C. albicans* was observed in any of the coral isolates. The highest and the second highest percentage of isolates displaying antimicrobial activity were observed in *Sarcophyton* sp. (sample J, 84.62 %) and *Nephtheidae* sp. (sample G, 20.29 %). Among the cultured corals, isolates from sample A (*Briareum* sp., 3.28 %) had the maximum percentage of isolates showing antimicrobial activity. No evidence of antimicrobial activity was found in the cultured corals *Nephtheidae* sp. (sample D) and *Sarcophyton* sp. (sample E), as well as the field-collected corals *Sarcophyton* sp. (sample H) and *Simularia* sp. (sample L).

The mean values and standard error of the percentage of antimicrobial isolates for each coral sample observed from each cultured and field-collected coral sample were  $1.53 \pm 0.79$  % and  $15.20 \pm 10.20$  %, respectively. A Mann–Whitney U test indicated that there was no difference in the percentage of antimicrobial bacteria between cultured ( $n = 4$ , excluding sample B) and field-collected isolates ( $n = 8$ ;  $P > 0.05$ ). However, the total average of the percentage of antimicrobial isolates for field-collected corals (8.6 %) was higher than for cultured corals (2.50 %).

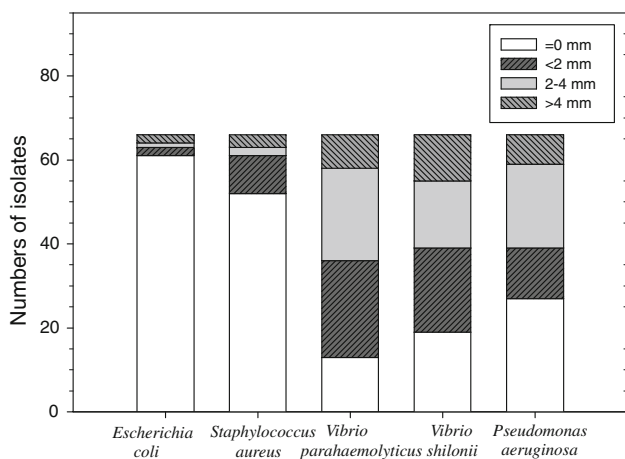
To further confirm and analyze the distribution of anti-bacteria activity, 66 isolates that showed an inhibition

**Table 2** Percentage (number) of bacterial strains exhibiting antimicrobial activity isolated from 13 soft corals using the agar block method

Sample	Genus of coral	Percentage of antimicrobial isolate	Percentage of anti- <i>V. shilonii</i>	Percentage of anti- <i>S. aureus</i>	Percentage of anti- <i>C. albicans</i>
A	<i>Briareum</i> sp.	3.28 (12)	2.19 (8)	1.09 (4)	0
B	<i>Briareum</i> sp.	0.83 (1)	0.83 (1)	0.00 (0)	0
C	<i>Lobophytum</i> sp.	3.60 (4)	1.80 (2)	2.70 (3)	0
D	<i>Nephtheidae</i> sp.	0.00 (0)	0.00 (0)	0.00 (0)	0
E	<i>Sarcophyton</i> sp.	0.00 (0)	0.00 (0)	0.00 (0)	0
F	<i>Briareum</i> sp.	9.37 (74)	5.95 (47)	5.95 (47)	0
G	<i>Nephtheidae</i> sp.	20.29 (14)	20.29 (14)	0.00 (0)	0
H	<i>Sarcophyton</i> sp.	0.00 (0)	0.00 (0)	0.00 (0)	0
I	<i>Sarcophyton</i> sp.	5.08 (3)	1.69 (1)	5.08 (3)	0
J	<i>Sarcophyton</i> sp.	84.62 (22)	69.23 (18)	80.77 (21)	0
K	<i>Sarcophyton</i> sp.	1.47 (2)	1.47 (2)	0.00 (0)	0
L	<i>Simularia</i> sp.	0.00 (0)	0.00 (0)	0.00 (0)	0
M	<i>Simularia</i> sp.	0.60 (1)	0.60 (1)	0.00 (0)	0
	Total	6.55 (133)	4.68 (95)	3.82 (78)	0



**Fig. 2** Distribution of the diameter of inhibition zone size for bacterial strains isolated from soft corals against five indicator bacteria using agar block method. The *first letter* of isolate indicates the coral sample (as in Table 1)



**Fig. 3** Distribution of the diameter of inhibition zone size for bacterial strains from soft corals using agar block method with antimicrobial activities against five indicator bacteria

zone > 1-mm diameter against at least one test strain were selected for secondary screening using agar block methods against six pathogens *E. coli*, *V. parahaemolyticus*, *P. aeruginosa*, *V. shilonii*, *S. aureus*, and *C. albicans* and the results were shown in Fig. 2 as well as Fig. 3. The results indicated that 80.30 % of these isolates possessed inhibitory activities against more than one indicator organisms (Fig. 2). 21.21 % inhibited Gram-positive

bacteria (*S. aureus*), 92.42 % inhibited Gram-negative bacteria (*E. coli*, *V. parahaemolyticus*, *V. shilonii* and/or *P. aeruginosa*), and 13.64 % inhibited both Gram-positive and Gram-negative bacteria. None of the isolates exhibited anti-*C. albicans* activity. Four isolates (6.06 %) were able to inhibit more than three indicator organisms. Most of antibiotic producer were isolated from the field-collected corals (87.88 %), such as *Briareum* sp. (50.00 %, sample L), *Nephtheidae* sp. (18.18 %, sample G), and *Sarcophyton* sp. (16.67 %, sample J).

The percentage of susceptibility of *E. coli*, *S. aureus*, *V. parahaemolyticus*, *V. shilonii*, and *P. aeruginosa* to the 66 coral isolates were 7.58, 21.21, 81.82, 71.21, and 59.09 % (Fig. 3), respectively. The percentage of susceptibility to the 66 coral isolates with inhibition zone diameters >4 mm were 3.03, 4.55, 12.12, 16.67, and 10.61 % for the aforesaid order of indicator bacteria. 19 isolates that showed an inhibition zone >4-mm diameter against at least one test strain or were able to inhibit at least four indicator organisms in secondary screening were selected for cytotoxic activity assays.

Cytotoxic activity assay

The cytotoxic activities of the crude extracts of all 19 isolates against two human cancer cell lines were tested (Table 3). Six isolates (31.6 %) were found to exhibit

**Table 3** The IC<sub>50</sub> of crude extract from 19 selected bacterial strains isolated from soft corals against the human promyelocytic leukemia cell line (HL-60) and the human T lymphoblastic leukemia cell line (CCRF-CEM) using the MTT method. Doxorubicin was used as the positive control

Isolate	IC <sub>50</sub> (μg/ml)	
	HL-60	CCRF-CEM
AGH8	–	–
AGH44	–	–
AGHN	–	–
AGH32A	–	–
AGH32B	–	–
AM56	–	–
CGH26	–	–
CGH28	30.36 ± 18.54	22.91 ± 15.41
CGH2XX	0.94 ± 0.59	1.19 ± 1.08
FGH406	85.14 ± 6.16	–
FGH440	68.28 ± 27.52	–
FGH455	–	–
FGH458	–	–
FGH462	–	–
FGH464	–	–
GGH9	–	–
JM33A	–	–
JM33B	92.90 ± 6.42	–
JM38	84.67 ± 51.85	42.86 ± 36.49
Doxorubicin	0.051 ± 0.074	0.0087 ± 0.0029

The IC<sub>50</sub>s were expressed as mean ± standard error

–, no activity

cytotoxic activity against the HL-60 tumor cell line, among which three of them also exhibited cytotoxic activity on CCRF-CEM cells.

The crude extract of a potent strain CGH2XX revealed high cytotoxic activity with IC<sub>50</sub> value 0.94 ± 0.59 and 1.19 ± 1.08 μg/ml against HL-60 and CCRF-CEM cell, respectively. Given that the extract of this isolate also inhibited the growth of a broad spectrum of bacteria (Fig. 2), the extract of this isolate was further tested for cytotoxic activity against normal PBMC. The IC<sub>50</sub> value of CGH2XX extract against normal PBMC was 243.7 ± 87.7 μg/ml and the stimulation index (SI) against HL-60 and CCRF-CEM cell was 259 and 204, respectively. The high stimulation index suggests that crude extract of CGH2XX has potential for antitumor treatment.

#### Comparison of bacterial growth in different media

We also compared the number of bacteria grown on marine agar, M1, GH, and GA, using cultured *Briareum* sp. as test sample. The results were expressed in CFU/g *Briareum* sp.

(means ± standard errors of the means): 2.38 × 10<sup>4</sup> ± 0.18 × 10<sup>4</sup>, 1.94 × 10<sup>4</sup> ± 0.44 × 10<sup>4</sup>, 1.89 × 10<sup>4</sup> ± 0.41 × 10<sup>3</sup>, and 3.09 × 10<sup>3</sup> ± 0.41 × 10<sup>3</sup> for the aforesaid order of media. The ratio of CFU/g *Briareum* sp. among the above media was 1: 0.815: 0.795: 0.130, respectively.

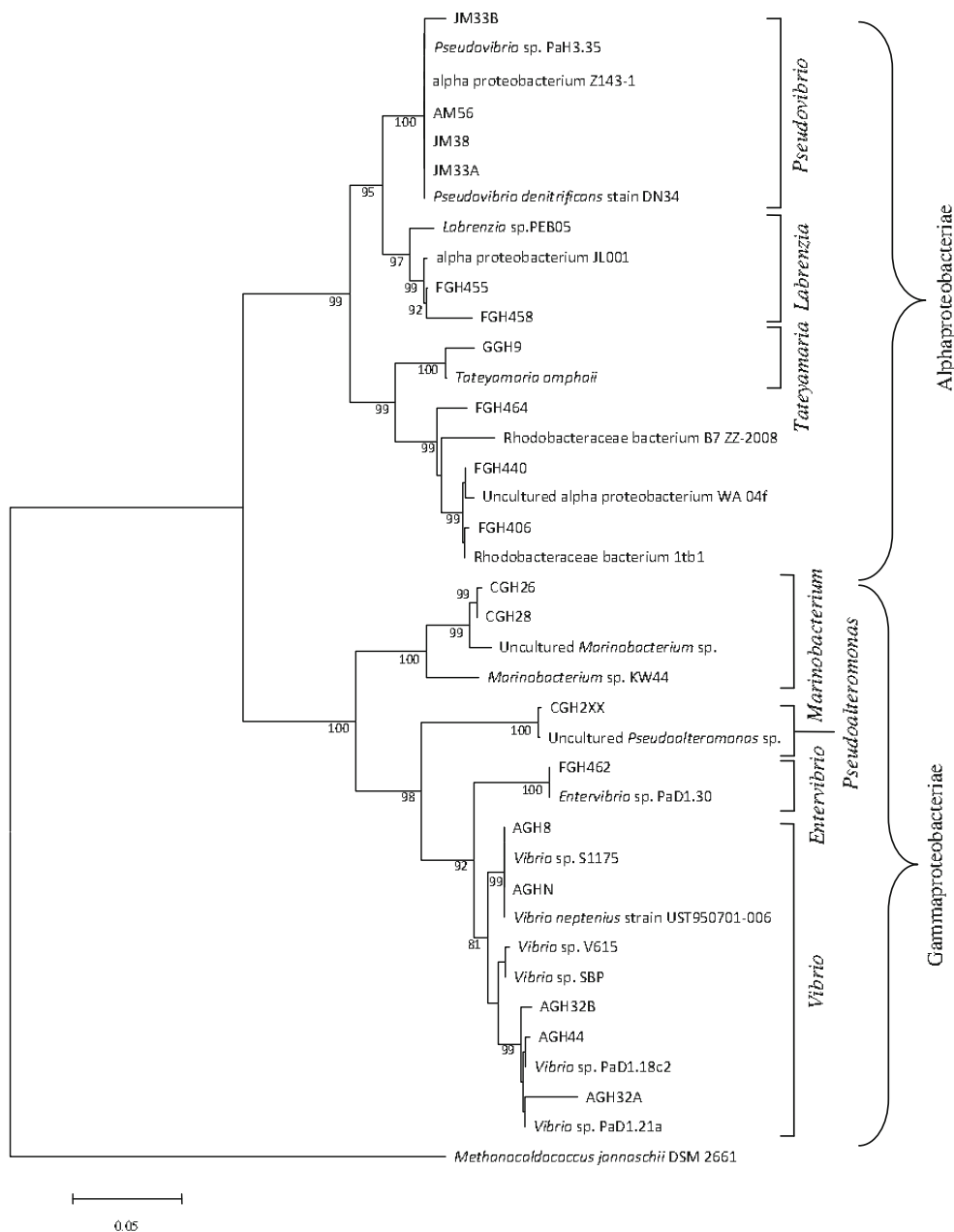
#### Identification of antibiotic producing bioactive bacterial isolates

16S rRNA genes sequences of the 19 isolates that exhibited high antimicrobial activities were sequenced and subsequently aligned to construct a phylogenetic tree (Fig. 4), based on around 650 bp sequences. These 19 selected isolates are, therefore, neither a representative sample of culturable bacteria nor a representative sample of culturable bacteria with antimicrobial activities of the coral. 19 isolates are belonging to the phylum Proteobacteria, among which 10 of them are of class Alphaproteobacteria whereas the remaining nine are of class Gammaproteobacteria (Fig. 4).

In addition, we also used Ribosomal Data Project (RDP) classifier tool (Cole et al. 2009) to classify bacterial based on their 16S rRNA sequences (Fig. 5). Among these 19 isolates, three isolates could not be classified to a known genus, but they are likely belonging to the family of Rhodobacteraceae (Fig. 4). The other 16 isolates belong to eight genera: *Vibrio* (5 isolates), *Pseudovibrio* (4), *Marinobacterium* (2), *Labrenzia* (2), *Pseudoalteromonas* (1), *Enterovibrio* (1), and *Tateyamaria* (1). Two genera, *Vibrio* and *Pseudoalteromonas*, were only isolated from cultured corals, whereas four genera, *Enterovibrio*, *Marinobacterium*, *Labrenzia*, and *Tateyamaria*, as well as three Rhodobacteraceae species were just isolated from field-collected corals. It is also interesting to note that all 10 Alphaproteobacteria species were all within family Rhodobacteraceae.

#### Discussion

Environmental factors, such as geographic and seasonal factors, have been reported to be some of the most important factors affecting the coral-associated bacterial community (Littman et al. 2009; Bourne et al. 2008). In this study, the culturable, heterotrophic bacteria inhabiting cultured and field-collected corals were compared using both the ratio of antibiotic and seawater-requiring isolates from cultured and field-collected corals as indices. Statistical analyses showed that there was no difference in the ratio of antibiotic and seawater-requiring isolates between cultured and field-collected corals, indicating that the difference in microbial communities associated with cultured and field-collected corals were negligible. Therefore, we



**Fig. 4** Phylogenetic tree of 19 selected bacterial strains isolated from the soft corals. The tree was constructed from a comparison of an approximately 650-bp region of the 16S rRNA gene sequence using the neighbor-joining analysis of a distance matrix with Kimura's two-parameter model. Bootstrap values (expressed as percentages of 100

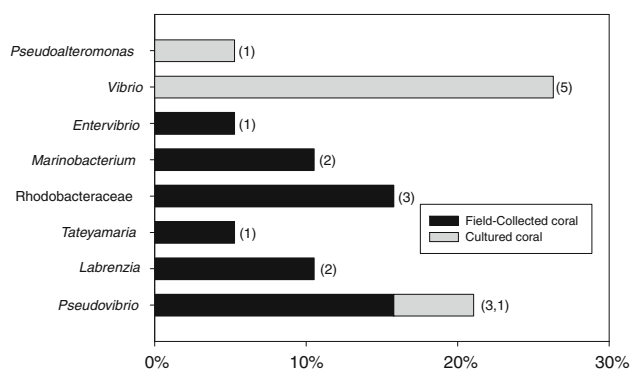
replications) more than 75 % are shown at branch points. The 16S rRNA sequence of *Methanocaldococcus jannaschii* DSM 2661 were used as outgroup. The scale bar represents 0.05 substitutions per nucleotide position

conclude that both cultured and field-collected soft corals could be used as a source for screening microorganisms with bioactive natural products. However, this result is based only on two indirect index of microbial flora. As such, further studies are required to analyze culturable bacterial flora between cultured and field-collected corals.

Several marine microbes can produce bioactive compounds, and in this study the cultured and field-collected soft corals were successfully used as a source to screen for

antibiotic-producing bacteria. Of a total of 2,030 isolates, 133 strains (6.55 %) showed antibiotic activity toward at least one indicator microbe used in the primary screening using agar block method. This observation is consistent with a previous study that 5.77 % of culturable bacteria isolated from the mucus of *Oculina patagonica* were antibiotic-producing strains (Nissimov et al. 2009). In contrast, the percentage of antibiotic-producing bacteria is lower than the observation by Shnit-Orland and Kushmaro





**Fig. 5** Taxonomic group percentage of the 19 selected bacterial strains isolated from the field-collected and cultured soft corals. Taxonomy was determined using classifier on the RDP database. Number in parentheses right after each bar indicates number of strains

(2009), in which about 25–27 % and 13 % of the bacterial isolates from the mucus of stony and soft coral possessed antimicrobial activity, respectively. Ritchie (2006) also showed that 20 % isolates from coral *Acropora palmata* were antibiotic producers. It was suggested that the percentage of antibiotic-producing strains can be affected by both the assay method as well as the species and the number of indicator microbes used in the screening (Long and Azam 2001; Shnit-Orland and Kushmaro 2009). For example, Gram-positive bacteria have been known to be more sensitive to antibiotic substances than Gram-negative bacteria (Nikaido 1996). Therefore, using more Gram-positive bacteria as indicator bacteria may lead to more antibiotic-producing bacteria. Nevertheless, our results suggest that soft coral can be a source for isolating marine bacteria producing antibiotics.

Several previous studies have supported the view that coral can be an untapped source of microbial diversity of economic importance (Shnit-Orland and Kushmaro 2009; Ritchie 2006). Therefore, evaluation of screening strategy, such as screening seawater dependent bacteria, may enhance the recovery rate of novel marine bacteria and improve our current understanding of antibiotic-producing coral bacteria (Mincer et al. 2002). A large fraction (total average 76.2 %) of our bacterial isolates displayed an obligate requirement of seawater for growth (Table 1). The requirement of seawater for bacterial growth suggests a high degree of marine adaptation or marine origin, and possibly little contamination from terrestrial sources. A previous study found that the percentage of seawater requiring bacteria were 57.7 % for isolates from the sponge *Haliclona simulans* (Kennedy et al. 2008), which is lower than our observations. However, it is interesting to note that our results agree with previous studies of seawater requirement of Gram-positive bacteria (about 82 %) in near-shore tropical marine samples (Jensen and Fenical 1995). Interestingly, the percentage of seawater-

requiring bacteria (33.43 %) from cultured *Briareum* sp. (sample B.) was found to be significantly lower than all the other coral (in Table 1). This result may have been the result of this coral species being diseased and suggests that there may be a large disparity concerning culturable bacterial flora between healthy and diseased coral, which agree with several previous studies that bacterial community of diseased or bleaching coral was significantly different from healthy coral (Koren and Rosenberg 2008; Luna et al. 2007).

The original objective of this study was to isolate marine bacteria with a focus on marine actinomycetes with antimicrobial activity from soft coral. Therefore, three actinomycete-selective media, M1, GH, and GA, were used to culture actinomycetes. However, we found that only few actinomycetal strains were cultured from soft coral, and none of them were seawater-requiring strains. Therefore, we decide to isolate bacteria from soft coral while still using these three media and compared them to the marine agar that is often used for marine bacteria isolation. Using cultured *Briareum* sp. as the sample, the ratios of CFU/g was 1: 0.815: 0.795: 0.130 among marine agar, M1, GH and GA, respective. The higher number of bacteria grown on M1 and GH indicates that they are both good media for isolation of bacteria with activity. However, it is possible that some species may behave differently among various media.

In Fig. 2, we found that the coral bacteria displayed different strength and spectra of activity against various indicator bacteria. The distribution of inhibition zone diameters for 66 antimicrobial bacterial strains from secondary screening (Fig. 3) showed a high percentage of inhibitive effects against pathogenic indicator bacteria from the ocean, namely *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa* and *Vibrio shilonii*, indicating the production of activities to inhibit the growth of specific marine microbial competitors. It is interesting to note that *Vibrio shilonii* has been shown to be a coral bleaching pathogen (Kushmaro et al. 1998) and are found in association with different coral species (Chimetto et al. 2008). All the above results suggest that the interactions between coral bacteria as well as host and coral bacteria could be diverse and complicated and different coral bacteria may contribute differently to the protection of the coral from marine pathogens (Nissimov et al. 2009). As pointed out by Shnit-Orland and Kushmaro (2009), these antibiotic-producing bacteria may play an important role in the first-line defense for coral against invading pathogens.

16S rRNA-based phylogenetic analysis was used to examine whether members of certain phylogenetic groups were more likely to possess antimicrobial activities. Our results showed that all of the antimicrobial isolates obtained from soft coral belonged to the phylum Proteobacteria, namely Alphaproteobacteria (53.6 %) and Gammaproteobacteria (47.4 %). Several previous studies have

displayed the predominance of Alphaproteobacteria and Gammaproteobacteria antimicrobial isolates from corals. For example, Nissimov et al. (2009) reported that production of antibiotics by marine isolates from the mucus of stony coral *Oculina patagonica* is due to Alphaproteobacteria (55.6 %) and Gammaproteobacteria (44.4 %). Comparing with several earlier works (Radjasa et al. 2008; Shnit-Orland and Kushmaro 2009), the bacterial diversity was low in this study. We think this is very possible the cost of screening antimicrobial bacteria that meet seawater requirement (Jensen and Fenical 1995). Another possible reason of this is that we only selected bacteria with strong antimicrobial activity for phylogenetic analysis.

Among all the samples, isolates from field-collected *Sarcophyton* sp. (sample L) displayed the highest percentage (82.81 %) of antimicrobial activity in the primary screening (Table 2). However, high variations in percentage of isolates displaying antimicrobial activity were observed for *Sarcophyton* sp. (sample E, H, I, and J) suggesting more test is need to confirm the percentage of antimicrobial isolates cultured from *Sarcophyton* sp. On the other hand, isolates from *Briareum* samples (except sample B) displayed high percentage of antimicrobial activity revealing that *Briareum* is a good source of antibiotic-producing bacteria.

For the isolate CGH2XX, its 16S rRNA gene BLAST results showed 98.3 % identity with *Pseudoalteromonas* sp. H02P24-23 (Genbank accession no. HQ161380). The phylogenetic tree also exhibited a similar relationship. The crude extracts from this red-pigmented isolate were found to have antimicrobial activity against three indicator microbes and cytotoxic activity against two cancer cells, plus low cytotoxic to normal PBMC. *Pseudoalteromonas* species have been known to develop metabolic pathways that produce biological compounds and antifouling agents (Wilson et al. 2010). In previous culture-based studies, Nissimov et al. (2009) as well as Shnit-Orland and Kushmaro (2009) isolated a number of *Pseudoalteromonas* species exhibiting antimicrobial activity from the mucus of coral species *Oculina patagonica* as well as *Pocillopora* sp., *Platygyra* sp., *Stylophora* sp., and *Porites* sp., respectively. Therefore, this bacterium is a good candidate for further natural product isolation and characterization research. In fact, we already isolated a new compound from the culture broth of CGH2XX (Chen et al. 2012).

Based on these results, we conclude that both cultured and field-collected soft coral could be used for discovery of marine bacteria producing new antimicrobial agents. Also, our study demonstrated that several bacterial strains isolated from soft coral demonstrated antimicrobial activity, suggesting that soft coral can be a source of natural antimicrobial agents; and these antibiotic-producing bacteria may also play an important role in the ecological interaction between

bacteria and their eukaryotic host. Following studies with these bioactive isolates will focus on natural product isolation and chemical structure identification in order to unravel their biotechnological potential.

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