

Antifouling activity and microbial diversity of two congeneric sponges *Callyspongia* spp. from Hong Kong and the Bahamas

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ABSTRACT: Microbial communities of the sponges *Callyspongia* sp. from Hong Kong and *Callyspongia plicifera* (Porifera: Demospongia) from the Bahamas were compared with each other and with those from reference substrata using a terminal restriction fragment length polymorphism (T-RFLP) analysis. The least number of bacterial ribotypes and bacterial isolates were retrieved from Bahamas reference and sponge surfaces, while the bacterial communities from Hong Kong *Callyspongia* sp. and reference surfaces were more diverse. Microbial communities from the 2 sponges were different from each other and from reference substrata. Gas chromatographic–mass spectrometric (GC-MS) analysis of dichloromethane extracts revealed that more than 60% of the compounds were similar in the 2 species *Callyspongia* sp. and *C. plicifera*, compared to the compounds of *Halichondria* spp. At tissue level (TL) concentrations, both sponge extracts predominantly inhibited the growth of bacteria from reference substrata. Multifactor ANOVA revealed that the source of bacteria (sponge surface, interior, or reference substrata), the geographic location of isolates (Hong Kong or the Bahamas), the sponge extract (from *Callyspongia* sp. or from *C. plicifera*), and combinations of these factors contributed significant effects in disc diffusion assay experiments. Sponge extracts at both TL concentrations and 10× dilutions were toxic to larvae of the polychaete *Hydroides elegans* and the barnacle *Balanus amphitrite*. Our results suggest that the 2 congeneric sponges *Callyspongia* spp. from different biogeographic regions have different bacterial associates, while producing relatively similar secondary metabolites. It remains to be explored whether differences in sponge-associated bacterial communities will also hold for other congeneric sponge species from different regions.

KEY WORDS: Sponge allelochemicals · Antifouling · Bacteria · Larvae · Microbial communities · Biogeographic comparison · South China Sea · Bahamas

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INTRODUCTION

Marine sponges (Porifera) are associated with a remarkable array of microorganisms that comprise actinomycetes (Burja & Hill 2001, Hill 2004) and other bacteria (Hentschel et al. 2001, Thoms et al. 2003), cyanobacteria (Thacker & Starnes 2003), fungi (Sponga et al. 1999), dinoflagellates (Burja & Hill 2001), and unicellular algae (Ruetzler 1985). These microorganisms are present on or inside most sponge species. Several

investigations have shown that microbiota from sponges differ from those associated with adjacent reference substrata and seawater (Santavy & Colwell 1990, Hentschel et al. 2002, Lee & Qian 2004, Dobretsov et al. 2005a), suggesting that these associations are specific and, perhaps, symbiotic.

Similar bacterial isolates were found in distantly related species of sponges (Webster & Hill 2001, Hentschel et al. 2002, Fieseler et al. 2004, Olson & McCarthy 2005). For example, Hentschel et al. (2002) demon-

strated that the sponges *Aplysina aerophoba* and *Theonella swinhoei* from the Mediterranean Sea, Red Sea and Pacific Ocean have a uniform microbial community that is phylogenetically different from that of marine plankton and marine sediments (Hentschel et al. 2002). *Chloroflexi* spp. bacteria have been found to be specific to all *Aplysina* species (Hentschel et al. 2003). Moreover, 3 major groups of Actinobacteria have been found in 3 species of *Xestospongia* spp. from different oceans (Montalvo et al. 2005). In contrast, Taylor et al. (2005) recently demonstrated that bacterial communities associated with the sponge *Cymbastela concentrica* from tropical waters differed from those in temperate Australia. However, the specificity of microbial associations from congeneric sponge hosts from different oceans and the specificity of bioactive metabolites from sponges remain largely unexplored.

Marine sponges are a rich source of unique and diverse bioactive metabolites (Faulkner 2000, Blunt et al. 2003). Many sponge- or sponge-symbiont-derived metabolites are potent antibacterial, antifungal, anti-feeding or antifouling compounds (Beccerro et al. 1997, Davis 1998, Bakus et al. 1986). Cytotoxic (Toth & Schmitz 1994), antibiotic (Wang et al. 1996) and anti-fouling (Tsukamoto et al. 1997) compounds were previously isolated from different representatives of *Callyspongia* spp. It has also been suggested that chemical defenses from tropical and temperate congeneric species of sponges are similarly strong and effective against predators (Becerro et al. 2003).

In this study we investigated the microbial community associated with 2 congeneric sponges *Callyspongia* spp. from the tropical western Atlantic (the Bahamas) and the western Pacific (Hong Kong) and tested the bioactivity of their extracts. Our objectives were specifically to: (1) compare the bacterial communities of 2 congeneric *Callyspongia* species and adjacent substrata, (2) compare the chemical profiles of dichloromethane extracts of these species with other sponge species, (3) examine the effects of crude extracts of these species on the growth of marine bacteria isolated from both sponges and from adjacent reference substrata, and (4) examine the effects of crude extracts on the larval attachment of dominant foulers in tropical waters—the polychaete *Hydroides elegans* and the barnacle *Balanus (Balanus) amphitrite*.

MATERIALS AND METHODS

Collection of sponges. Specimens of the sponge *Callyspongia* sp. were collected from Hong Kong at depths of 1 to 3 m, at the pier next to the Coastal Marine Laboratory (CML) of the Hong Kong University of Science and Technology (22°22'N, 114°16'E) in

August 2004. In their natural habitats, these yellow-white solitary sponges are free from fouling and attach to underwater structures of the pier, and are surrounded by clumps of oysters. The sponges were identified by Prof. Rob van Soest, and voucher specimens were placed in the Zoological Museum of Amsterdam under the archive number ZMAPOR 17598. The sponge *Callyspongia plicifera* (Lamarck, 1814) was collected from Great Stirrup Cay, Bahama Islands (25°51'N, 77°53'W) at a depth of about 5 m during a field survey conducted by the research vessel RV 'Steward Johnson I' from the Harbor Branch Oceanographic Institution, Florida, in July 2003. These fluorescent-blue or purple-pink sponges have no biofouling and inhabit coral reefs. The sponge *C. plicifera* can grow solitarily or in groups. For the chemical comparison of crude extracts (see below), the sponge *Halicondria* sp. (ZMAPOR 17603) was collected from Hong Kong waters from the same pier next to the CML and the sponge *H. melanodocia* was collected from Great Stirrup Cay, Bahama Islands.

In all cases, sponges were carefully brought to the water surface by SCUBA divers and transferred to large buckets. Wet weight was measured and the tissue volume was determined by water displacement in a graduated cylinder filled with seawater.

Bacterial isolation. Prior to the isolation of bacteria the sponges were soaked several times in autoclaved 0.22 µm-filtered seawater (AFSW) in order to wash away bacteria that were not associated with sponges and might represent contaminants from the water column. The outer surfaces of 6 sponges were swabbed with 5 separate sterile cotton balls for the collection of cultivable epibiotic bacteria. To collect bacteria from the sponge interior, sponges were cut with a sterile scalpel and internal surfaces were swabbed with sterile cotton balls. Three sample swabs from 5 sponge individuals were combined and suspended in AFSW from the location of each species (Hong Kong or the Bahamas) and from the sponge exterior and interior. Bacterial suspensions were diluted (0.1 and 0.01×) in AFSW. Aliquots of 200 µl of each suspension were streaked onto nutrient agar (composed of 1.5% agar in 0.22 µm filtered seawater (FSW), 0.3% yeast extract, 0.5% peptone) with replication (n = 3) and incubated at 30 ± 1°C under a 15:9 h light:dark photoperiod. After 1 to 3 d of incubation, bacterial colonies were examined under a dissecting microscope for conspicuous characteristics such as colour, shape, size, surface topography, and the presence of granules. Distinguishable colonies were isolated and purified. Bacteria were also isolated from reference substrata (stones and shells) within a range of 0.5 m of the sponges. These bacterial isolates were stored in 50% glycerol at –80°C. Bacterial strains were identified by compara-

tive analysis of their 16S rRNA gene sequences as described in Lau et al. (2002). The primers used in the PCR were 355F forward (5'-ACTCCTACGGGAG-GCAGC-3') (Amann et al. 1990), 1055R reversal (5'-CACGAGCTGACGACAGCCAT-3') (Lee et al. 1993), 926F forward (5'-CCGTCAATTCCTTTRAGTTT-3') (Lee et al. 1993) and 1492R reversal (5'-GGYTACCCT-GTTACGACTT-3') (Eden et al. 1991). Fragments of DNA sequences obtained from individual primers were assembled using the Sequencher® software package (Gene Codes). The closest match to the 16S rRNA gene sequence of the respective bacterium was retrieved by comparison with data from GenBank (www.ncbi.nlm.nih.gov). The extent of similarity between the bacterial isolate sequence and a GenBank close match sequence was based on the percent of sequence identity.

Bacterial communities of the sponges and adjacent surfaces. In order to compare bacterial communities, 3 outer and inner surfaces (20 cm²) of the sponge and 3 outer surfaces of reference substrata (stones in close proximity to the sponge) were swabbed with sterile cotton balls following the method described by Lee & Qian (2004). After each swab the cotton balls were individually suspended in 1 ml extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M sodium chloride, 1% CTAB; at pH 8) in 2 ml micro-centrifuge tubes. The samples were lysed by 3 cycles of freezing and thawing followed by 2 h incubation in 20% sodium dodecylsulfate (SDS) at 65°C. The cotton balls were removed and after centrifugation (10000 rpm × 5 min⁻¹), the total DNA in the supernatant was extracted and purified in a volume of 24:1 chloroform:isoamyl-alcohol, followed by precipitation in isopropanol at room temperature for 15 min. The precipitated DNA was washed with cold (-20°C) 70% ethanol, dried and re-suspended in 50 µl of autoclaved double-distilled water and frozen until use.

Polymerase chain reaction (PCR) of 16S rRNA genes of bacterial communities was performed in a total volume of 25 µl containing 1 µl of DNA template, 250 µM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotechnology), 1 U of DNA Taq polymerase (Amersham Biosciences) and 0.8 µM of each universal primer: 341F forward (5'-CCTACGGGAGGCAGCAG-3') and 926R reversal (5'-CCGTCAATTCCTTTRAGTTT-3') (Amann et al. 1990, Lee et al. 1993). The 926R primer was labeled at the 5' end with 6-carboxy fluorescein (FAM) dye. The thermocycling conditions were as follows: 95°C for 2 min (1 cycle); 95°C for 30 s (15 cycles), 60°C for 3 min and 72°C for 3 min. The annealing temperature started at 60°C and was reduced to 45°C in increments of 1°C cycle⁻¹; 10 cycles of 95°C for 30 s, 45°C for 3 min and

72°C for 3 min; and 72°C for 10 min. Amplified DNA (4 µl of PCR mixtures) was visualized by gel electrophoresis on a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer.

Fluorescently labeled PCR products were purified with the Wizard® PCR preps DNA purification system (Promega) according to the manufacturer's protocol. Purified amplicons were digested with 20 U *MspI* (Boehringer Mannheim Biochemicals) at 37°C for 6 h. Aliquots of digested products (10 µl) were mixed with 0.5 µl of internal size standard (ET550-R, Amersham Biosciences). This mixture was denatured for 2 min at 95°C and immediately chilled on ice prior to capillary electrophoresis on a MegaBACE™ genetic analyzer (Amersham Biosciences) operated in the genotyping mode. After electrophoresis, the lengths of the fluorescently labeled terminal restriction fragments (T-RFs) were determined by comparison with internal size standards using the 'Fragment Profiler' software (Amersham Biosciences). The lengths of T-RFs obtained by the analyzer were rounded up to the nearest integral values. Peaks that were less than 1.5 bp apart from a larger peak were classified as its 'shoulders' and thus eliminated (Dunbar et al. 2001). For each sample, peaks over a threshold of 50 fluorescence units (Blackwood et al. 2003) and whose peak heights contributed at least 1% to the integrated height (Buchan et al. 2003, Luna et al. 2005) were used for analysis. Terminal fragments <35 bp and >500 bp were excluded from the analysis to avoid detection of primers and uncertainties of size determination. In order to relate 16S rRNA sequences of isolates with the length of particular T-RFLP peaks, we used NEBcutter analysis (<http://tools.neb.com/NEBcutter2/index.php>) that allowed us to mimic the digestion of the rRNA sequence by the *MspI* enzyme.

Extraction of sponges and chemical profiles of sponge extracts. In the laboratory, 5 sponge specimens belonging to the same species (*Callyspongia* sp., *C. plicifera*, *Halichondria* sp. or *H. melanodocia*) were combined together and extracted 3 times with dichloromethane (DCM) for 8 h by gentle agitation. The extracts were filtered through a paper filter (Whatman No. 1) and concentrated by rotary evaporation to a concentration that represented 10× tissue level (TL). In the case of *Callyspongia* sp. and *C. plicifera*, the concentration of crude extracts was adjusted prior to each experiment (see below). Prior to larval bioassay experiments, extracts were evaporated until dry, and subsequently re-dissolved in dimethyl sulfoxide (DMSO) (Sigma). Before the experiments, extracts were mixed with AFSW and screened at TL (1×), 10-fold stronger (10×) (disk diffusion bioassay), 10-fold weaker (0.1×) and 100-fold weaker (0.01×) (larval bioassay) concentrations.

Crude extracts of *Callyspongia* sp. and *Halichondria* sp. from Hong Kong, and *C. plicifera* and *H. melanodocia* from the Bahamas were dried under vacuum, re-dissolved in DCM and subsequently separated and analyzed by coupled gas chromatography–mass spectrometry (GC-MS) with electron impact ionization (Varian CP-3800, ion trap Varian-2200). GC-MS separations were performed on relatively non-polar capillary columns (CP-Sil 8 CB-MS, 30 m length, 0.25 μm film thickness, 0.25 mm i.d.). The injection port was held at 275°C and the interface at 300°C. The temperature was programmed from 75°C to 310°C at 10°C min^{-1} . Helium was used as the carrier gas. Each peak on a chromatogram represents an individual compound that was separated from the extract. In order to identify compounds from extracts we used the National Institute of Standards and Technology (NIST) GC-MS library. The library compares mass spectra of unknown individual compounds with those in the library. The closest match (with the highest probability) to the library was recorded. Peaks over a threshold of 20 k counts were used for the analysis. DCM was used as a control and peaks that appeared on the control chromatogram were excluded from the analysis.

Antibacterial assay. In order to investigate antibacterial effects of sponge extracts from the Hong Kong sponge *Callyspongia* sp. and the Caribbean sponge *C. plicifera*, DCM extracts at 1 \times and 10 \times TL concentrations were pipetted onto circular paper discs (Whatman No. 1; disc volume 20 μl). An additional set of discs loaded with 40 μg disc^{-1} of streptomycin and with 20 μl DCM were used as an antibiotic control and a solvent control correspondingly. Each bacterial strain was inoculated onto agar plates, and the dried discs, either with the sponge extracts or with the controls, were then placed onto the plates. Agar plates were incubated for 24 h at 30°C. The experiments were performed with 5 replicates. The observed zones of growth inhibition between the disc and the bacterial film were measured to the nearest 0.2 mm by a stereomicroscopic inspection.

Larval bioassays. Adults of the tubeworm *Hydroides elegans* (Haswell) were collected from submerged rafts of the fish farm at Wong Shek, Hong Kong (22° 25' N, 114° 20' E). Larval cultures were prepared and maintained according to Bryan et al. (1997). Only competent larvae were included in the bioassays. Metamorphic competence of *H. elegans* larvae was determined by their characteristic morphology and by a competency bioassay with the larval attachment stimulator 3-isobutyl-1-methylxanthine (IBMX, Fluka) at 10⁻⁴ M in AFSW (Lau & Qian 1997, Pechenik & Qian 1998).

Adults of the barnacle *Balanus amphitrite* (Pitombo) were collected from stones at Marina Cove (22° 21' N,

114° 17' E). Standard procedures were used to obtain and rear *B. amphitrite* larvae (Thiyagarajan et al. 2002). Nauplii were reared in batch cultures on a mixed diet (1:1) of *Chaetoceros gracilis* Schutt (LB 2658, UTEX) and *Skeletonema costatum* (Greville) (CCMP 1332) at approximately 1000 larvae l^{-1} AFSW. The initial algal concentration was set at 2.5 $\times 10^5$ cells ml^{-1} and kept constant subsequently. Cyprids were aged for 4 d in darkness at 8°C prior to attachment bioassays (Rittschof et al. 1992).

Each larval bioassay was conducted with replication ($n = 6$) in multi-well polystyrene dishes (#3047, Falcon) containing sponge extracts (1 \times or 10 \times concentrations) and 20 larvae of *Hydroides elegans* or *Balanus amphitrite*. Multi-well dishes filled with 1 ml AFSW and containing 4% DMSO solution were used as controls. The IBMX (Fluka) at 10⁻⁴ M in FSW was used as an artificial stimulator of larval attachment for *H. elegans* (Dobretsov & Qian 2002). *Hydroides elegans* larvae were pre-treated with 10⁻⁴ M IBMX for 30 min and then used for the larval bioassay. Both larval attachment assays were run at 24°C under continuous illumination for 24 h. After this period of time, swimming, attached, and dead larvae were counted using a dissecting-microscope.

Statistical analysis. The diameter of inhibition zones of bacterial growth produced by different sponge extracts were square-root transformed prior to a multi-factor ANOVA (Zar 1999) in order to investigate the effect of the extract source (*Callyspongia* sp. or *C. plicifera*), site of bacterial origin (sponge interior, exterior, or adjacent substratum), and geographic location (Hong Kong or the Bahamas), of isolates using results of the disk-diffusion bioassay. When no inhibition zone was produced, a value of 0.5 was given to improve normality of the data distribution (Zar 1999). For each larval bioassay, the differences between control and treatment were determined by a Dunnett's *t*-test (Zar 1999). Prior to this analysis the percentages of settled larvae and dead larvae were arcsine transformed (Zar 1999). In the cases of zero attached or dead larvae, a value of $\frac{1}{4}n$ ($n =$ number of larvae in a single replicate) was given to improve the normality of the data distribution (Zar 1999). In all cases, the normality assumption was verified by the Shapiro-Wilk test (Shapiro & Wilk 1965). T-RF patterns of different bacterial community DNA samples were subjected to cluster analysis. Bray-Curtis similarities were used to produce a similarity matrix based on the total number of T-RFs observed in all samples and the presence or absence of these T-RFs in individual samples. For the construction of a dendrogram demarcating the similarity of microbial communities in the gels, group average linkage in a hierarchical, agglomerative clustering algorithm was performed using the PRIMER program (Plymouth Marine Labora-

tory) (Clarke & Warwick 1994). Chemical profiles of sponges crude extracts were subjected to cluster analysis. We constructed a Bray-Curtis similarity matrix based on the total number of peaks presented in all extracts and the presence or absence of these T-RFs in individual samples. A dendrogram showing the similarity of crude extracts was constructed using the PRIMER program. In all cases, the threshold level for significance was 95%.

RESULTS

Bacterial isolates from sponges and reference substrata

Only 7 bacterial strains were obtained from the exterior and interior of the Bahamas sponge *Callyspongia plicifera* and 9 strains from adjacent substrata (Table 1). Seven strains from the Bahamas sponges and from the adjacent substrata belonged to the γ -Proteobacteria group. Bacteria belonging to the *Firmicutes* group were found only among spongal isolates, and not from any of the nearby reference substrata from the Bahamas. One bacterial isolate from a Bahama reference surface belonged to the *Schinkobacteria* group. Most of the bacteria from Hong Kong reference substrata belonged to the *Firmicutes*- and γ -Proteobacteria (Table 1). None of the bacterial isolates from reference substrata belonged to the γ -Proteobacteria or the *Actinobacteria*. Bacteria from the exterior and interior of Hong Kong *Callyspongia* sp. predominantly belonged to the γ -Proteobacteria.

Bacterial communities associated with sponges and adjacent substrata

The least number of bacterial ribotypes (T-RFs = 13 to 22) were found on Bahamian samples, both from sponges and from adjacent substrata, while double the number of T-RFs (i.e. ribotypes) were found on Hong Kong samples (Table 2). Bacterial communities associated with sponges and reference substrata were characterized by the absence of certain ribotypes and the presence of other ribotypes (Table 2). Some T-RFs were unique to certain types of substrata (i.e. T-RF at 40 bp in the Bahama reference, 431 bp and 455 bp in the Hong Kong reference; 176 bp, 307 bp for *C. plicifera* and 184 bp, 199 bp for *Callyspongia* sp.) or to certain locations (i.e. 422 bp in the Bahamas, 47 bp and 179 bp T-RFs in the Hong Kong samples). In contrast, T-RFs of 246 bp were present in all substrata, suggesting that 1 or several bacterial species are widely distributed on a variety of different substrata and in

different oceans. Both sponges showed unique T-RFs (i.e. 41 bp, 310 bp and 419 bp), suggesting that some bacteria are uniquely associated with certain sponge species.

NEBcutter analysis allowed us to correlate particular bacterial isolates with the presence of particular T-RFs. Most of the isolates did not match with any of the T-RFs. The T-RF (246 bp) that was present in all T-RFLP profiles matched with the sequence of a *Firmicutes* sp. isolate (Hkr7) from the Hong Kong reference location. A T-RF of 199 bp observed in *Callyspongia* sp. corresponded to the internal isolate (Hki9) from this sponge. A T-RF of 119 bp that was present in all sponges matched with the sequence of an internal isolate (Hki8) from *Callyspongia* sp.

According to Bray-Curtis similarity matrices, based on the presence (indicated by 1) or absence (indicated by 0) of a given T-RF, the bacterial communities were divided into several distinct groups (Fig. 1). In particular, both the exterior and interior bacterial communities associated with both species of *Callyspongia* were different from bacterial communities found on reference substrata. Bacterial communities obtained from the exterior and interior surfaces of sponges *Callyspongia* sp. and *C. plicifera* were different from each other and formed separate clusters in the dendrogram (Fig. 1). Bacterial communities from adjacent substrata collected in Hong Kong were highly variable in bacterial composition, as indicated by a low similarity between reference replicates.

Chemical profile of crude extracts from *Callyspongia* spp.

The chemical profiles of crude extracts from *Callyspongia* spp. from the Bahamas and Hong Kong were

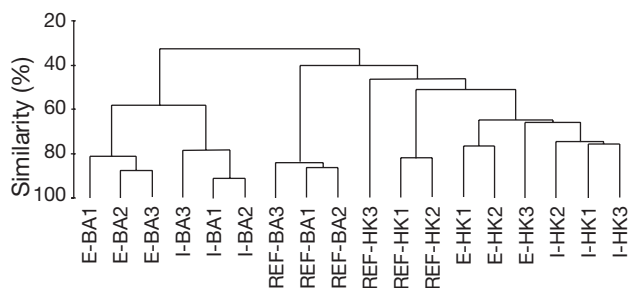


Fig. 1. Cluster analysis indicating similarities (%) among bacterial communities obtained from exterior (E) and interior (I) tissue of sponges *Callyspongia* sp. (Hong Kong, HK) and *Callyspongia plicifera* (the Bahamas, BA) and reference sites (stones close to the sponge; REF-HK and REF-BA, correspondingly). Different replicates (n = 3) identified by corresponding numbers

Table 1. Phylogenetic status of bacterial isolates from sponges *Callyspongia plicifera* (the Bahamas) and *Callyspongia* sp. (Hong Kong) and reference surfaces. 16S rRNA gene sequences of individual bacterial isolates were compared to nucleotide sequences in GenBank. The closest matching nucleotide sequence for each bacterial isolate is indicated by strain name, accession number and percent of sequence similarity

| Code | UST accession no. | Closest match in GenBank (accession no.) | Similarity (%) | Location |
|---|-------------------|---|----------------|----------------------|
| Proteobacteria, γ-Proteobacteria | | | | |
| Care321 | UST030701-192 | <i>Balneatrix alpica</i> Y17112 | 91 | Bahamas, exterior |
| Care319 | UST030701-192 | <i>Pseudomonas elongate</i> AF500006 | 95 | Bahamas, exterior |
| Care314 | UST030701-188 | <i>Pseudoalteromonas porphyrae</i> AY771715 | 97 | Bahamas, exterior |
| Carr480 | UST030701-333 | <i>Alteromonas marina</i> AF529060 | 98 | Bahamas, substrata |
| Carr507 | UST030701-362 | <i>Alteromonas marina</i> AF529060 | 99 | Bahamas, substrata |
| Carr501 | UST030701-068 | <i>Alteromonas marina</i> AF529060 | 96 | Bahamas, substrata |
| Carr495 | UST030701-348 | <i>Pseudoalteromonas ruthenica</i> AF316891 | 95 | Bahamas, substrata |
| Hke13 | UST040911-013 | <i>Vibrio harveyi</i> AY750578 | 99 | Hong Kong, exterior |
| Hke12 | UST040911-012 | <i>Alteromonas macleodi</i> Y18228 | 98 | Hong Kong, exterior |
| Hke6 | UST040911-006 | <i>Alteromonas macleodi</i> Y18228 | 99 | Hong Kong, exterior |
| Hke5 | UST040911-005 | <i>Alteromonas marina</i> AF529060 | 98 | Hong Kong, exterior |
| Hke4 | UST040911-004 | <i>Alteromonas macleodi</i> subsp. <i>fijiensi</i> AJ414399 | 98 | Hong Kong, exterior |
| Hke3 | UST040911-003 | <i>Alteromonas marina</i> AF529060 | 99 | Hong Kong, exterior |
| Hke9 | UST040911-009 | <i>Marinobacter aquaeolei</i> AJ000726 | 99 | Hong Kong, exterior |
| Hke2 | UST040911-002 | <i>Psychrobacter</i> sp. ANT9171 AY167289 | 98 | Hong Kong, exterior |
| Hki20 | UST040911-035 | <i>Vibrio tubiashi</i> X74725 | 97 | Hong Kong, interior |
| Hki18 | UST040911-033 | <i>Vibrio natriegens</i> X74714 | 99 | Hong Kong, interior |
| Hki17 | UST040911-032 | <i>Vibrio fischeri</i> AY292941 | 99 | Hong Kong, interior |
| Hki16 | UST040911-031 | <i>Vibrio harveyi</i> AY750578 | 99 | Hong Kong, interior |
| Hki15 | UST040911-030 | <i>Vibrio corallilyticus</i> AJ440004 | 96 | Hong Kong, interior |
| Hki13 | UST040911-028 | <i>Vibrio harveyi</i> AY750578 | 99 | Hong Kong, interior |
| Hki12 | UST040911-027 | <i>Vibrio harveyi</i> AY750578 | 99 | Hong Kong, interior |
| Hki10 | UST040911-025 | <i>Vibrio corallilyticus</i> AJ440004 | 99 | Hong Kong, interior |
| Hki8 | UST040911-023 | <i>Vibrio hollisae</i> AJ514911 | 96 | Hong Kong, interior |
| Hki6 | UST040911-021 | <i>Vibrio corallilyticus</i> AJ440004 | 99 | Hong Kong, interior |
| Hki4 | UST040911-019 | <i>Vibrio harveyi</i> AY264925 | 97 | Hong Kong, interior |
| Hki2 | UST040911-017 | <i>Vibrio fischeri</i> AY292941 | 99 | Hong Kong, interior |
| Hkr11 | UST040911-046 | <i>Alteromonas macleodii</i> Y18228 | 99 | Hong Kong, substrata |
| Hkr10 | UST040911-045 | <i>Vibrio harveyi</i> X74693 | 98 | Hong Kong, substrata |
| Hkr9 | UST040911-044 | <i>Alteromonas macleodii</i> AMY18228 | 99 | Hong Kong, substrata |
| Hkr8 | UST040911-043 | <i>Alteromonas macleodii</i> Y18228 | 97 | Hong Kong, substrata |
| Hkr6 | UST040911-041 | <i>Vibrio probioticus</i> AJ345063 | 95 | Hong Kong, substrata |
| Hkr3 | UST040911-038 | <i>Vibrio aestuarianus</i> AJ845014 | 94 | Hong Kong, substrata |
| α-Proteobacteria | | | | |
| Cari307 | UST030701-185 | <i>Pseudovibrio denitrificans</i> AY486423 | 99 | Bahamas, interior |
| Cari298 | UST030701-181 | <i>Pseudovibrio denitrificans</i> AY486423 | 99 | Bahamas, interior |
| Carr515 | UST030701-031 | <i>Pseudovibrio denitrificans</i> AY486423 | 99 | Bahamas, substrata |
| Carr504 | UST030701-359 | <i>Erythrobacter flavus</i> AF500005 | 98 | Bahamas, substrata |
| Carr491 | UST030701-344 | <i>Erythrobacter flavus</i> strain SW-46 AF500004 | 99 | Bahamas, substrata |
| Carr479 | UST030701-331 | <i>Erythrobacter flavus</i> strain SW-52 AF500005 | 100 | Bahamas, substrata |
| Hki19 | UST040911-034 | <i>Ruegeria atlantica</i> D88527 | 96 | Hong Kong, interior |
| Hki9 | UST040911-024 | <i>Silicibacter lacuscaerulensis</i> U77644 | 97 | Hong Kong, interior |
| Firmicutes, Bacillales | | | | |
| Care308 | UST030701-186 | <i>Bacillus hwajinpoensis</i> AF541966 | 98 | Bahamas, exterior |
| Cari299 | UST030701-182 | <i>Bacillus megaterium</i> AF142677 | 98 | Bahamas, interior |
| Hke11 | UST040911-011 | <i>Exiguobacterium</i> sp. AY744448 | 95 | Hong Kong, exterior |
| Hke10 | UST040911-010 | <i>Exiguobacterium marinum</i> AY594266 | 97 | Hong Kong, exterior |
| Hke 8 | UST040911-008 | <i>Exiguobacterium gaetbuli</i> AY594264 | 98 | Hong Kong, exterior |
| Hke 7 | UST040911-007 | <i>Planococcus citreus</i> AF500008 | 98 | Hong Kong, exterior |
| Hki7 | UST040911-022 | <i>Bacillus</i> sp. ROO40 AY188840 | 99 | Hong Kong, interior |
| Hki3 | UST040911-018 | <i>Bacillus megaterium</i> AF142677 | 99 | Hong Kong, interior |
| Hki1 | UST040911-016 | <i>Bacillus cereus</i> ZK CP000001 | 98 | Hong Kong, interior |
| Hkr16 | UST040911-050 | <i>Bacillus thuringiensis</i> serovar <i>konkukian</i> AE017355 | 99 | Hong Kong, substrata |
| Hkr15 | UST040911-049 | <i>Bacillus cereus</i> ZK CP000001 | 99 | Hong Kong, substrata |
| Hkr14 | UST040911-048 | <i>Bacillus cereus</i> ATCC 14579 AE017013 | 97 | Hong Kong, substrata |
| Hkr7 | UST040911-042 | <i>Exiguobacterium marinum</i> AY594266 | 98 | Hong Kong, substrata |
| Hkr4 | UST040911-039 | <i>Bacillus megaterium</i> AJ717381 | 97 | Hong Kong, substrata |
| Hkr2 | UST040911-037 | <i>Bacillus cereus</i> ATCC 14579 AE17013 | 99 | Hong Kong, substrata |
| Hkr1 | UST040911-036 | <i>Bacillus cereus</i> ZK CP000001 | 99 | Hong Kong, substrata |
| Bacteroides, Schingobacteria | | | | |
| Carr492 | UST030701-345 | <i>Microscilla furvescens</i> AB078079 | 97 | Bahamas, substrata |
| Actinobacteria, Actinobacteridae | | | | |
| Hki14 | UST040911-029 | <i>Micrococcus luteus</i> AJ717367 | 99 | Hong Kong, interior |
| Hki11 | UST040911-026 | <i>Micrococcus</i> sp. Ellin149 AF408991 | 99 | Hong Kong, interior |
| Hki5 | UST040911-020 | <i>Micrococcus luteus</i> AJ717369 | 98 | Hong Kong, interior |

Table 2. T-RFLP profiles of bacterial communities obtained from exterior (E) and interior (I) tissues of sponges *Callyspongia* sp. (Hong Kong, HK) and *C. plicifera* (the Bahamas, BA) and reference substratum from the near vicinity (REF-HK and REF-BA, correspondingly). The presence and fragment sizes (bp) of individual T-RFs are denoted as follows: X^a = present in 3 out of 3 replicates; X^b = present in 2 out of 3 replicates (presence in 1 out of 3 replicates is omitted). Blank cell = absence of particular T-RF in a given biofilm

| T-RF (bp) | E-BA | I-BA | REF-BA | E-HK | I-HK | REF-HK | T-RF (bp) | E-BA | I-BA | REF-BA | E-HK | I-HK | REF-HK |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 40 | | | X ^a | | | | 208 | | | | X ^a | X ^a | X ^a |
| 41 | X ^b | X ^a | | X ^a | X ^a | | 209 | | X ^b | | | X ^a | X ^a |
| 42 | | | | | X ^b | | 215 | | | X ^a | | | |
| 44 | | | | | X ^a | X ^a | 219 | | | | X ^a | | |
| 45 | | | | X ^a | | X ^b | 221 | | | | X ^a | | |
| 46 | | | | | | X ^b | 222 | | | | | X ^a | |
| 47 | | | | X ^b | X ^a | X ^b | 242 | | | | | X ^a | |
| 48 | | | | X ^b | | X ^b | 246 | X ^a | X ^a | X ^b | X ^a | X ^b | X ^b |
| 69 | | | | X ^a | X ^b | | 247 | | | X ^a | | | |
| 71 | | | X ^a | X ^b | | X ^a | 248 | | | | X ^a | X ^a | X ^b |
| 73 | | | X ^b | | | | 298 | | X ^a | | | X ^b | |
| 74 | | | X ^a | X ^b | | | 299 | | X ^b | X ^a | X ^a | X ^a | X ^a |
| 75 | | | X ^a | X ^a | X ^a | | 300 | | X ^a | X ^a | X ^a | | |
| 77 | | | | | | X ^a | 301 | | | X ^a | | | X ^a |
| 79 | | | | | | X ^b | 302 | | | X ^a | X ^a | X ^a | X ^a |
| 85 | | X ^a | | | | | 307 | X ^a | X ^a | | | | |
| 89 | | | X ^b | X ^a | X ^b | | 309 | X ^b | | | X ^a | X ^a | |
| 91 | | | | | | X ^a | 310 | X ^a | X ^a | | X ^b | X ^a | |
| 93 | | | | X ^b | X ^b | | 312 | | X ^b | X ^a | | | X ^a |
| 100 | | | | | X ^a | | 313 | | | X ^a | X ^b | | X ^a |
| 109 | | X ^a | | | | | 314 | | | | | X ^b | X ^a |
| 119 | | X ^a | | X ^b | X ^b | | 315 | | | | X ^a | | |
| 121 | | | | | X ^a | | 364 | | X ^a | | | | |
| 122 | | | | X ^b | X ^a | | 369 | X ^b | X ^a | | X ^b | X ^a | |
| 123 | | X ^a | | | | | 370 | | X ^a | | X ^a | X ^a | X ^a |
| 126 | | | | X ^b | | X ^b | 371 | | | | X ^a | X ^a | X ^b |
| 127 | | | | X ^a | X ^a | | 373 | | | X ^a | | | |
| 130 | | | | X ^a | X ^b | | 374 | X ^a | | | | | X ^b |
| 147, 150 | | | | | X ^b | | 375 | X ^a | X ^a | | | | X ^a |
| 176 | X ^a | X ^a | | | | | 376 | | | | X ^a | X ^a | X ^b |
| 177 | | X ^a | | X ^b | | | 377 | | | | | X ^a | |
| 179 | | | | X ^a | X ^a | X ^b | 400 | | X ^b | | | | |
| 180 | | | X ^a | X ^a | | X ^a | 416 | X ^b | | | | | |
| 182 | | | X ^a | X ^a | X ^a | X ^a | 417 | X ^b | | | | | |
| 184 | | | | X ^b | X ^a | | 419 | X ^a | X ^a | | X ^b | X ^a | |
| 199 | | | | X ^a | X ^a | | 422 | X ^a | X ^b | X ^a | X ^b | X ^a | |
| 202 | | X ^a | | X ^b | | | 423 | | | | X ^a | X ^b | X ^b |
| 204 | | | | | X ^a | X ^a | 424 | | | | X ^b | | |
| 205 | | | | X ^a | | X ^a | 431 | | | | | | X ^b |
| 207 | | | | | | X ^b | 455 | | | | | | X ^a |
| | | | | | | | Total number | 13 | 23 | 19 | 43 | 40 | 35 |

very similar compared with extracts of *Halichondria* spp. from the same locations (Fig. 2, Table 3). More than 60% of compounds from the congeneric sponges *Callyspongia* sp. and *C. plicifera* were similar, while the percentage similarity between extracts of *Halichondria* sp. and *H. melanodocia* was noticeably lower. Some peaks (i.e. peak with retention time 14.9 min) were present in all sponges, indicating that Demospongiae from different phylogenetic groups may have similar secondary metabolites (Table 3). Some metabolites (i.e. peaks with retention times of 6.5, 13.1 and 14.3 min) were specific only to congeneric species of

sponges but there were also specific secondary metabolites that were present only in particular species of sponges (i.e. peaks with retention times of 3.1, 5.1, 5.6 and 16.0 min).

Antibacterial assay

Extracts of both *Callyspongia* sp. and *C. plicifera* inhibited the growth of bacterial isolates from the Bahamas reference site at TL and 10× TL concentrations (Fig. 3A). At TL concentration, the crude extracts

Table 3. Gas chromatography–mass spectrometry (GC-MS) analysis of dichloromethane extracts of *Callyspongia* sp. and *Halichondria* sp. from Hong Kong, and *C. plicifera* and *H. melanodocia* from the Bahamas. X = presence of peaks; blank cells = absence of peaks. Peaks were identified by NIST GC-MS library; compounds with a probability <20% to closest match with library marked as unknown

| Peak retention time (min) | Extract | | | | Close match to the library | Probability % |
|---------------------------|-------------------------|---------------------|-------------------------|-----------------------|---|---------------|
| | <i>Callyspongia</i> sp. | <i>C. plicifera</i> | <i>Halichondria</i> sp. | <i>H. melanodocia</i> | | |
| 3.1 | | | X | | Unknown | <20 |
| 3.2 | | | X | | Disiloxane, 1, 3-bis(chloromethyl)-1,1,3,3-tetramethyl | 55 |
| 3.4 | | | X | | Unknown | <20 |
| 3.5 | | | X | | Propanoic acid, 3-(trimethylsilyl), ethyl ester | 44 |
| 5.1 | X | | | | 1,3,3-trimethoxybutane | 75 |
| 5.4 | | | X | | Pyridine, 3-trimethylsiloxy- | 74 |
| 5.6 | X | | | | Pyridine, 2,4,6-trimethyl- | 48 |
| 5.6 | | | | X | Silanol, trimethyl carobonate (2:1) | 61 |
| 5.8 | | | X | X | Propanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester | 33 |
| 6.2 | | | X | | Methyl 2-[2-(4-chlorophenyl)-5-methyl-1H]imidazol-1-yl]dithiobenzoate | 34 |
| 6.3 | | | | X | Pentaneodioic acid., 2-[(trimethylsilyl)oxy]-, dimethyl ester | 53 |
| 6.5 | X | X | | | Unknown | <20 |
| 7.2 | | | | X | Arabino-hexos-2-ulose, 3,4,5,6-tetrakis-O-(trimethylsilyl)-, bis(dimethyl acetal) | 73 |
| 7.3 | X | | | | 2-ethyl-6-isopropyl pyridine | 62 |
| 7.5 | | | | X | Benzoic acid trimethylsilyl ester | 42 |
| 7.6 | | | X | | Pentasiloxane dodecamethyl- | 78 |
| 8.1 | X | X | | | Silane, cyclohexyldimethoxymethyl- | 92 |
| 8.6 | X | | | | Pyridine, 2,4,6-trimethyl- | 39 |
| 8.8 | X | | | | Pentasiloxane dodecamethyl- | 73 |
| 8.9 | | | X | | Trimethylsilyl ether of glycerol | 75 |
| 9.2 | X | X | | X | Alpha-D-galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)- cyclic methylboronate | 22 |
| 9.4 | X | X | | | Unknown | <20 |
| 9.7 | X | X | | | Hexyl octyl ether | 22 |
| 10.7 | | | X | | Aminomalonic acid, tris(trimethylsilyl)- | 94 |
| 10.8 | X | | | | Hexasiloxane tetradecamethyl | 96 |
| 11.2 | | | | X | Creatinine enol tri-TMS | 90 |
| 11.6 | | | X | | n-octanoic acid, 2-[(trimethyl)amino]-, trimethylsilyl ester | 25 |
| 12.2 | X | X | | X | 3,5-di-tert-butyl-4-trimethylsilyloxytoluene | 84 |
| 12.5 | X | X | | | Phenol 2,4,bis(1,1-dimethyl)- | 40 |
| 12.6 | X | | | | Heptasiloxane hexadecamethyl | 95 |
| 12.8 | | | X | | Myo-inositol, 5-deoxy-1,2,3,4,6-pentakis-O-(trimethylsilyl)- | 24 |
| 13.1 | | | X | X | Cyclononasiloxane, octadecamethyl | 83 |
| 13.4 | | | X | | Unknown | <20 |
| 13.8 | | | X | | Unknown | <20 |
| 13.9 | | | | X | Hexadecanoic acid, trimethylsilyl ester | 82 |
| 14.3 | X | X | | | 1-octanol 2 butyl | 22 |
| 14.6 | X | | | | Methyl 12-methyl-tridecanoate | 88 |
| 14.9 | X | X | X | X | Arachidonic acid | 25 |
| 15.0 | X | | | | Methyl 12-methyl-tridecanoate | 43 |
| 15.1 | | | X | X | Palmitelacidic acid, trimethylsilyl ester | 88 |
| 15.2 | | | | X | Hexadecanoic acid, trimethylsilyl ester | 75 |
| 15.4 | | | X | | Unknown | <20 |
| 15.5 | | | | X | Unknown | <20 |
| 15.8 | | | | X | Cyclononasiloxane, octadecamethyl | 72 |
| 15.9 | X | | | | Unknown | <20 |
| 16.0 | | X | | | Methyl palmitate | 80 |
| 16.1 | X | X | | | 11-hexadecenoic acid methyl ester | 46 |
| 16.2 | X | | | | 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | 57 |
| 16.3 | X | X | | | 3-acridinol, 9-phenyl | 43 |
| 16.6 | X | X | | | Unknown | <20 |

Table 3 (continued)

| Peak retention time (min) | <i>Callyspongia</i> sp. | Extract | | | Close match to the library | Probability % |
|---------------------------|-------------------------|---------------------|-------------------------|-----------------------|---|---------------|
| | | <i>C. plicifera</i> | <i>Halichondria</i> sp. | <i>H. melanodocia</i> | | |
| 16.9 | X | X | | | Hexadecanoic acid 15 methyl ester | 38 |
| 17.0 | | X | X | | 5-hexadecenoic acid 2-methoxy methyl ester | 23 |
| 17.1 | | | X | X | 9,12-octadecanoic acid (Z,Z)-, trimethylsilyl ester | 26 |
| 17.2 | X | X | X | | Octadecanoic acid eicosyl ester | 21 |
| 17.3 | | | | X | 11-trans-octadecenoic acid, trimethylsilyl ester | 28 |
| 17.5 | | | | X | Cyclododecasiloxane, eicosamethyl- | 66 |
| 17.7 | X | X | | | 13-octadecenoic acid methyl ester Z | 23 |
| 17.8 | X | | X | | 1-dotriacontanol | 22 |
| 17.9 | | X | | | Methyl octadecanoate | 68 |
| 18.2 | X | X | | | 2-hexadecenoic acid 2,3,-dimethyl methyl ester E | 24 |
| 18.3 | | | X | | Unknown | <20 |
| 18.4 | | | X | | Cephalotaxine, 11-(acetyloxy)-, acetate (ester), (11. Alpha) | 25 |
| 18.7 | | X | | X | Octadecanoic acid 14-methyl methyl ester | 29 |
| 18.9 | | | | X | 1-monolinoleoylglycerol trimethylsilyl ether | 29 |
| 19.0 | | X | | | Tetrahydrariaucarolone | 55 |
| 19.1 | | X | | | 3-oxatricyclo[0.8.0.0(7,16)]tricyclopenta-1(22), 7(16),9,13,23,29-hexaene | 38 |
| 19.2 | | X | | | 14-oxononadec-10-enoic acid methyl ester | 22 |
| 19.3 | X | X | | | Nonadecanoic acid 10-methyl methyl ester | 46 |
| 19.5 | | X | | | Unknown | <20 |
| 19.9 | X | X | | | Heneicosanoic acid methyl ester | 96 |
| 20.1 | | | | X | Cyclododecasiloxane, eicosamethyl- | 57 |
| 20.2 | X | | X | | Unknown | <20 |
| 20.3 | X | | | | Unknown | <20 |
| 20.4 | X | | X | | Unknown | <20 |
| 20.5 | X | | | | Tricyclo[20.8.0.0(7,16)]tricyclopentane, 1(22), 7(16)-diepoxy | 28 |
| 20.7 | X | X | | | Unknown | <20 |
| 20.8 | X | X | | | Unknown | <20 |
| 21.4 | | X | | | 13-octadecenal (Z) | 24 |
| 21.6 | | X | | | Docosahexaenoic acid 1,2,3,-propanetriyl ester | 22 |
| 21.8 | | X | | X | E,E,Z-1,3,12-Nonadecatriene-5,14-diol | 24 |
| 21.9 | | X | | | 15-tetracosenoic acid, methyl ester (Z) | 64 |
| 22.0 | X | X | | | Tetracosanoic acid | 89 |
| 22.3 | X | | | | Benzenedodecanoic acid, 3-methoxy-2-(methoxycarbonyl)-methyl ester | 24 |
| 22.4 | | | X | | Unknown | <20 |
| 22.5 | X | X | | | Squalene | 28 |
| 22.6 | X | | | | Pentacosanoic acid methyl ester | 90 |
| 22.9 | X | X | | | Unknown | <20 |
| 23.2 | X | X | | | Hexacosanoic acid methyl ester | 55 |
| 23.3 | | X | | | Unknown | <20 |
| 23.5 | | X | | | 26,27-dinoregost-5-ene-3,24-diol, (3.beta) | 39 |
| 24.2 | X | X | | | 5,14,23,-octadecatrien-14,15-diol | 22 |
| 24.4 | X | X | | | Cholesta-5,22-dien-3-ol,(3beta) | 21 |
| 24.7 | X | X | | | 17-(1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetra | 61 |
| 25.0 | X | X | | | Unknown | <20 |
| 25.3 | | | | X | Unknown | <20 |
| 25.4 | X | X | | | 26,26-dimethyl-5,24(28)-ergostadien-3.beta-ol | 25 |
| 25.5 | X | X | | | Campesterol | 23 |
| 25.7 | X | X | | | Stigmasterol | 62 |
| 26.2 | | X | | | Beta-sitosterol | 42 |
| 26.4 | X | X | | | Sigmasta-5,24(28)-dien-3-ol, (3beta) | 42 |
| 26.5 | | | X | | Pregn-5-ene-3,11,20-trione | 27 |
| 27.0 | X | X | | | 26,26-dimethyl-5,24(28)-ergostadien-3.beta-ol | 30 |
| 27.6 | | | | X | Unknown | <20 |
| 28.6 | | X | | | Lycoxanthin | 42 |
| 29.0 | | X | | | Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-octadecyl ester | 64 |

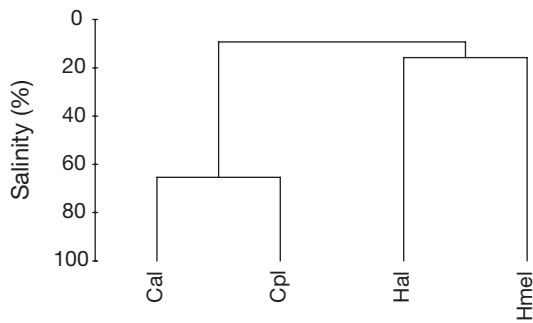


Fig. 2. Cluster analysis indicating similarities (%) among crude extracts of sponges *Callyspongia* sp. (Hong Kong, Cal), *C. plicifera* (the Bahamas, Cpl), *Halichondria* sp. (Hong Kong, Hal) and *H. melanodocia* (the Bahamas, Hmel)

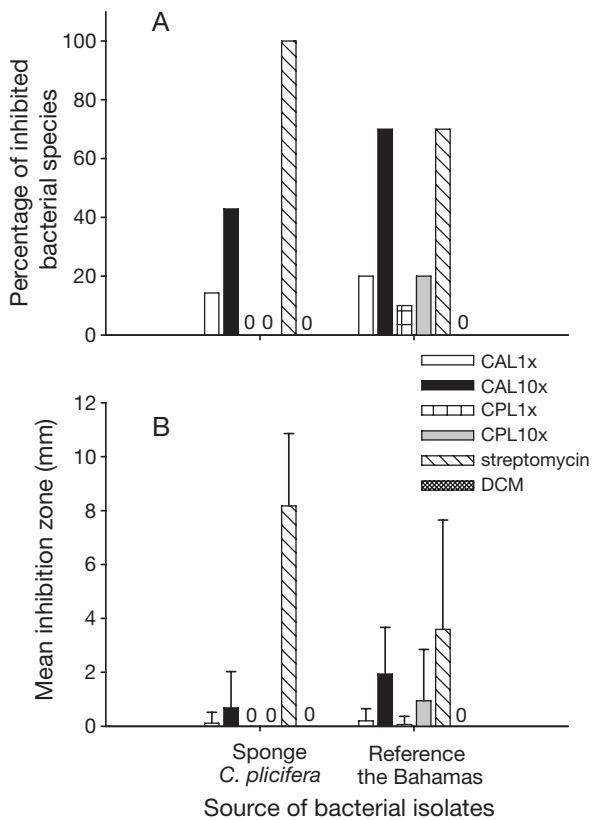


Fig. 3. Inhibition of growth of bacterial isolates from sponge *Callyspongia plicifera* and reference substrata from the Bahamas. In total, 9 isolates from reference substrata and strains from *C. plicifera* were tested. (A) Percentage of bacterial isolates inhibited by crude extracts of *Callyspongia* sp. (CAL) and *C. plicifera* (CPL) at tissue level (TL; 1×) and 10 times TL (10×) concentrations. (B) Mean ± SD of inhibition zones (mm) in disc diffusion bioassays with crude extracts of *Callyspongia* sp. and *C. plicifera* at TL (1×) and 10 times TL (10×) concentrations. Streptomycin 40 µg disc⁻¹ and dichloromethane (DCM; 20 µg disc⁻¹) used as controls. Due to low numbers, bacterial isolates from *C. plicifera* were combined into 1 sample; 0 = no inhibition of growth of a particular group of bacterial isolates

had much less of an antibiotic effect on reference bacteria from the Bahamas than the streptomycin control, whereas the crude extracts of *Callyspongia* sp. at 10× TL concentration level were as inhibitory as the streptomycin control for reference bacteria (Fig. 3A,B). Extracts of *C. plicifera* did not inhibit the growth of isolates from this sponge, while extracts of *Callyspongia* sp. had little inhibitory effect on the growth of these bacteria. The DCM control did not affect bacterial growth (Figs. 3 & 4).

At TL concentration and 10× TL concentration, the extracts of both *Callyspongia* sp. and *C. plicifera* effectively inhibited the growth of reference bacteria from Hong Kong, while the strains associated with the interior surface of *Callyspongia* sp. were less sensitive to sponge extracts (Fig. 4). The extracts of *Callyspongia* sp. at TL concentration did not affect the growth of bacteria from the interior of this sponge. In contrast,

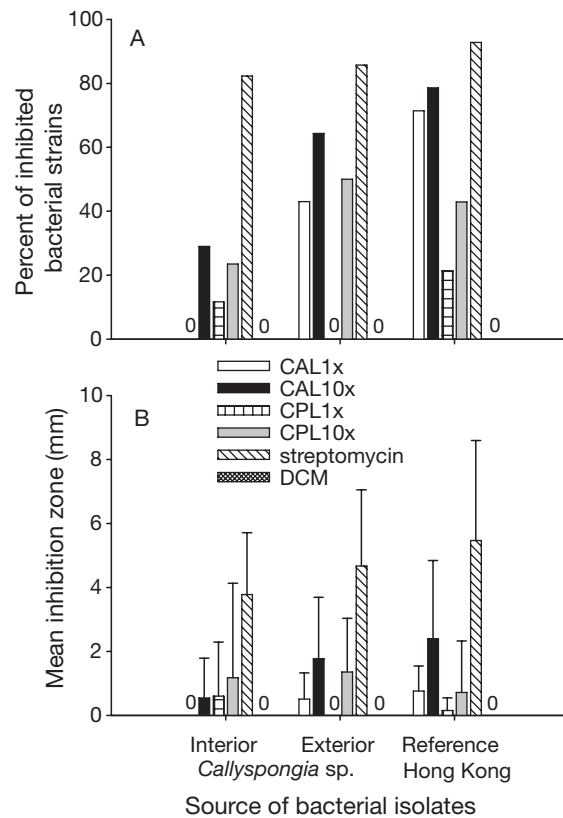


Fig. 4. Inhibition of growth of bacterial isolates from sponge *Callyspongia* sp. and reference substrata from Hong Kong. (A) Percentage of bacterial isolates inhibited by crude extracts of *Callyspongia* sp. (CAL) and *C. plicifera* (CPL) at TL (1×) and 10 times TL (10×) concentrations. (B) Mean ± SD of inhibition zones (mm) in a disc diffusion bioassay with crude extracts of *Callyspongia* sp. and *C. plicifera* at TL (1×) and 10 times TL (10×) concentrations. Streptomycin at µg disc⁻¹ and DCM (20 µg disc⁻¹) used as controls; 0 = no inhibition of growth of a particular group of bacterial isolates

the extracts of *C. plicifera* inhibited the growth of bacteria from both internal and reference substrata at TL concentration but did not affect the growth of external isolates. The least amount of inhibition was observed for sponge extracts at TL concentration, while the inhibition zones for 10× concentrated extracts were similar to those produced by the streptomycin control (Fig. 4B).

In general, the streptomycin control had a more potent effect on bacterial isolates than crude extracts of both sponge species at TL concentration, while the effect of sponge crude extracts at 10× TL concentration were not much different from those of the streptomycin control (Figs. 3 & 4). Moreover, the growth of some bacteria such as α -Proteobacteria (Carr491, Carr479), *Firmicutes* (Hkr16), and γ -Proteobacteria (Carr501, Hke13) was not affected by streptomycin but was inhibited by sponge extracts.

Multifactor ANOVA revealed that the inhibition of bacterial growth in the disc diffusion bioassays with extracts of sponges at TL concentration varied with the geographic location of the bacterial isolates tested (Hong Kong or the Bahamas) and with sponge extract (*Callyspongia* sp. or *C. plicifera*) (Table 4). The location, the source of bacteria (interior, exterior and reference) and sponge extracts in combination affected the results of the disk diffusion experiments. At 10× TL concentration of extracts, both the source of bacteria and the extract affected bacterial growth. We found that combinations of factors (the habitat source of bacteria and bacterial geographic origin, as well as the source of bacteria [sponge or reference substrata] and the extracts) affected the results of the disc diffusion experiments at 10× TL concentration of extracts.

Larval bioassay

The extracts of both sponge species affected larval attachment and larval mortality of *Hydroides elegans* and *Balanus amphitrite* (Fig. 5). The extracts from *Callyspongia* sp. and *C. plicifera* at TL concentration were toxic to larvae of the polychaete *H. elegans* and the barnacle *B. amphitrite* (Fig. 5B,D). A 100× diluted concentration of both sponge extracts did not show any effect on larval mortality. Only the extracts of *Callyspongia plicifera* at 10× dilution inhibited larval attachment of *B. amphitrite* (Fig. 5A).

DISCUSSION

In our investigation, the T-RFLP analysis demonstrated that microbial communities associated with *Callyspongia* sp. and *Callyspongia plicifera* sponges

Table 4. Multifactor ANOVA. Effects of bacteria source (exterior, interior, and substrata), geographic location (Hong Kong, the Bahamas) and sponge extract (*Callyspongia* sp. and *C. plicifera*) on the inhibition of bacterial growth. Data were square root transformed prior to analysis (n = 5)

| Effects | df | MS | F | p |
|---|-----|--------|--------|--------|
| Crude extracts at tissue level concentration | | | | |
| Source | 2 | 0.930 | 1.574 | 0.208 |
| Location | 1 | 4.722 | 7.988 | 0.004 |
| Sponge | 1 | 2.722 | 4.606 | 0.032 |
| Source × Location | 2 | 0.527 | 0.891 | 0.411 |
| Source × Sponge | 2 | 6.312 | 10.678 | <0.001 |
| Species × Location | 1 | 0.003 | 0.006 | 0.937 |
| Source × Location × Sponge | 2 | 3.424 | 5.792 | 0.003 |
| Residual | 618 | 0.591 | | |
| Crude extracts at 10× tissue level concentration | | | | |
| Source | 2 | 73.828 | 19.431 | <0.001 |
| Location | 1 | 0.897 | 0.236 | 0.627 |
| Sponge | 1 | 18.927 | 4.981 | 0.026 |
| Source × Location | 2 | 15.468 | 4.071 | 0.018 |
| Source × Sponge | 2 | 33.335 | 8.773 | 0.0002 |
| Extract × Location | 1 | 0.054 | 0.014 | 0.905 |
| Source × Location × Sponge | 2 | 5.178 | 1.362 | 0.257 |
| Residual | 618 | 3.800 | | |

were different from microbial communities developed on the reference substrata in close proximity to the sponges. This fact was further supported by the existence of some bacterial isolates specifically associated with sponges. These findings suggest that sponges can directly (by the production of chemical compounds) or indirectly (by having inhibitive symbionts) modify the bacterial communities associated with them. Similar results have been shown in previous experiments with proliferating sponge primmorphs (Thakur et al. 2003). Lee & Qian (2003, 2004) also demonstrated that bacterial isolates and bacterial communities found on the surface of the sponge *Mycale adhaerens* were different from communities found on inanimate reference surfaces. Further, similar observations were made for internal microbial communities of sponges (Burja & Hill 2001, Hentschel et al. 2002, Taylor et al. 2004, 2005), indicating that sponges have distinct bacterial communities.

Microbial communities associated with *Callyspongia* sp. were more diversified than those associated with *C. plicifera*, as the number of T-RFs (ribotypes) from Hong Kong was higher than that from the Bahamas. For the first time we were able to demonstrate clear differences in bacterial communities between 2 congeneric sponges from the West Atlantic (the Bahamas) and the West Pacific (Hong Kong). This contradicts the assumption that similar bacterial communities are associated with sponges from different seas (Hentschel et al. 2002, 2003, Fieseler et al. 2004, Olson &

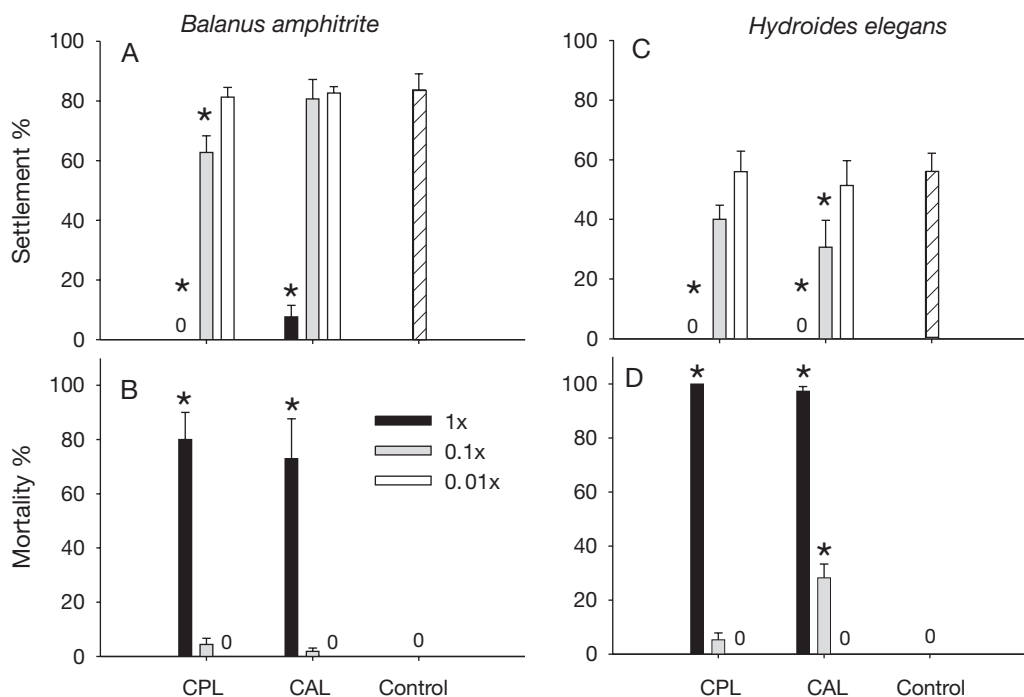


Fig. 5. (A, B) Barnacle *Balanus amphitrite* and (C, D) polychaete *Hydroides elegans* larval attachment and mortality in the presence of crude extracts of *Callyspongia* sp. from Hong Kong (CAL) and *C. plicifera* from the Bahamas (CPL) at TL (1x), 10-fold dilution (0.1x) and 100-fold dilution (0.01x). *Significantly different ($p < 0.05$) from the control (FSW with 3% DMSO) according to Dunnett's *t*-test. Results = mean \pm SE; 0 = no attachment (A & C) or no mortality (B & D) of larvae in experiments

McCarthy 2005). However, our results are in a good agreement with the findings of Taylor et al. (2005), who also showed that bacterial communities associated with the sponge *Cymbastela concentrica* from tropical waters differed from those in temperate Australia, whereas bacterial communities over a 500 km range were similar. Of course, it remains to be explored whether differences in sponge-associated bacterial community structures will also hold for other sponge species from different geographic regions.

GC-MS analysis demonstrated that more than 60% of the compounds from the Bahamas *Callyspongia plicifera* and the Hong Kong *Callyspongia* sp. were similar compared to *Halichondria* species from the same locations. Taking into account that microbial communities of *Callyspongia* spp. were different, this finding may suggest that most of the extracted chemical compounds are synthesized by the sponges and that they are specific to *Callyspongia* spp. Van Soest & Braekman (1999) suggested referring to these as 'taxonomically distributed compounds'.

What kinds of 'taxonomically distributed compounds' are known for *Callispongiidae* spp.? Surprisingly, the information about the presence of similar chemical compounds in sponges is limited (Erpenbeck & van Soest 2005). Most compounds, like peroxide-containing acids (Toth & Schmitz 1994), pyridine alka-

loids (Wang et al. 1996) and terpenes (Tsukamoto et al. 1997), are unique and isolated only from particular *Callyspongia* species. In contrast, 3-alkylpiperidine derivatives have a dominant presence in *Callyspongia* spp. and their distribution may be congruent with the phylogenetic affinities of sponges (Andersen et al. 1996). None of the above-mentioned compounds were found during our experiment. There are 2 possible explanations for this. Firstly, most of the isolated compounds have been identified through biomedical studies; therefore, they may be unique to and present in particular sponge species only. Secondly, the *Callyspongia* genus contains >200 species, which have a paraphyletic or polyphyletic origin according to their molecular phylogeny (van Soest & Braekman 1999). Therefore, in this large group of sponges, related species may not have exactly the same secondary metabolites. We therefore suggest investigation of the chemical composition and production of secondary metabolites by congeneric species from different geographic locations to test this assumption.

In the disc diffusion bioassay, crude extracts of both *Callyspongia* species predominantly inhibited the growth of bacteria isolated from the reference surfaces, but inhibited the bacterial isolates from the sponges only to a minor extent, suggesting that bioactive compounds could have played a role in the selec-

tive retention of bacteria in sponge tissue. Notably, the growth of only 1 out of 20 bacterial isolates from the sponge *Mycale adhaerens* was affected by sponge extracts (Lee & Qian 2004), and sponge extracts selectively inhibited bacterial attachment and growth in assays by Kelly et al. (2003). Similarly, Kelman et al. (2001) found that extracts of the sponge *Amphimedon viridis* did not inhibit the growth of sponge-associated bacteria, but did affect the growth of bacteria isolated from the water column. Extracts of the sponges *Pachychalina* sp., *Acanthella cavernosa*, and *Xestospongia testudinaria* altered bacterial community profiles on hydrogel surfaces, suggesting that the attachment of some bacteria was prevented during the period of experiments with running seawater (Harder et al. 2004). Newbold et al. (1999) reported that different species of Caribbean sponges produced different antibacterial compounds that targeted different bacterial strains. Thus, the specific effects of sponge extracts on surface bacteria indicate a potential role of sponge metabolites in the formation of sponge-specific microbial communities.

The source of bacteria (sponge exterior, interior, or adjacent substrata), the geographic location of bacteria (Hong Kong or the Bahamas), the extracts (*Callyspongia* sp. or *C. plicifera*), and a combinations of these factors all had statistically significant effects on the results of the disc diffusion experiments. The present study, for the first time, investigated the effect of sponge extracts on bacterial isolates from congeneric sponge species from different geographic locations. The following studies are the only examples that share similar lines of experimentation with this study: Assmann et al. (2000) found that concentrations of the anti-predator compound scyprin from the sponges *Agelas wiedenmayeri* and *A. conifera* from different locations of the Bahama Islands were different. Green (1977) found that bioactivity and toxicity in the marine sponges *Haliclona* spp. and *Halichondria* spp. from different latitudes on the Pacific side of the North American continent were different, indicating that toxicity in sponges increased with decreasing latitude. Green (1977) also found that sponges exposed to predators (fishes) produced a wider range of anti-feeding substances. Therefore, it is prudent to expand this sort of comparative study in order to achieve a better understanding of ecological roles of bacteria associated with sponges.

In our experiments, the extracts of *Callyspongia* sp. and *C. plicifera* at TL concentration and at 10× dilutions were toxic to larvae of the polychaete *Hydroides elegans* and the barnacle *Balanus amphitrite*. In fact, toxicity of sponges to marine organisms has been well documented (Becerro et al. 1997, Amsler et al. 2000, Lee & Qian 2003, Dobretsov et al. 2004, 2005a,b). For

instance, spherulous cells of the encrusting sponge *Crambe crambe* produce toxic guanidine alkaloids with antifouling, antipredation, and allelopathic functions in nature (Beccerro et al. 1997). The sponge *Mycale adhaerens* inhibits larval attachment of *H. elegans* by excreting toxic waterborne compounds (Lee & Qian 2003). Conditioned seawater from the sponge *Callyspongia* (*E.*) *pulvinata* is also toxic to the benthic diatom *Nitzschia paleacea* and larvae of the tube worm *H. elegans* (Dobretsov et al. 2004). It is possible that similar toxic metabolites exist in other species of *Callyspongiidae*, including the 2 sponge species studied here.

Overall, this study demonstrated that 2 congeneric *Callyspongia* species from 2 biogeographically different zones accommodated distinct bacterial communities but produced relatively similar secondary metabolites compared to other sponge species. This result should be taken into account in future investigations where a better understanding of sponge microbial associations needs to be developed—including an understanding of their dynamics and potential to produce bioactive metabolites—in order to interpret this invertebrate-microbe association more realistically.

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