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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-

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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, antifeeding or antifouling compounds (Beccerro et al. 1997, Davis 1998, Bakus et al. 1986). Cytotoxic (Toth & Schmitz 1994), antibiotic (Wang et al. 1996) and antifouling (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 *Halichondria* 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 cm^2) 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 desoxyribonucleotide 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 *Msp*I (Boehringer Mannheim Biochemicals) at 37°C for 6 h. Aliquots of digested products (10 µl) were mixed with 0.5 µµl 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 *Msp*I 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× and 10× 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 2.5×10^5 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× or 10× 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 multifactor 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 ¼ 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 *Schingobacteria* 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 *Callypongia* spp.

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

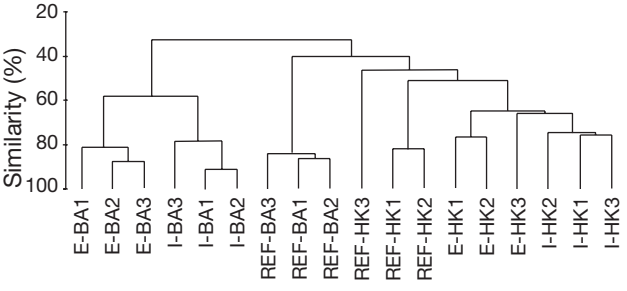

Fig. 1. Cluster analysis indicating similarities (%) among bacterial communities obtained from exterior (E) and interior (I) tissue of sponges *Callyspongia* sp. (Hong Kong, HK) and *Callyspongia plicifera* (the Bahamas, BA) and reference sites (stones close to the sponge; REF-HK and REF-BA, correspondingly). 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	*Balneatrix alpica* Y17112	91	Bahamas, exterior
Care319	UST030701-192	*Pseudomonas elongate* AF500006	95	Bahamas, exterior
Care314	UST030701-188	*Pseudoalteromonas porphyrae* AY771715	97	Bahamas, exterior
Carr480	UST030701-333	*Alteromonas marina* AF529060	98	Bahamas, substrata
Carr507	UST030701-362	*Alteromonas marina* AF529060	99	Bahamas, substrata
Carr501	UST030701-068	*Alteromonas marina* AF529060	96	Bahamas, substrata
Carr495	UST030701-348	*Pseudoalteromonas ruthenica* AF316891	95	Bahamas, substrata
Hke13	UST040911-013	*Vibrio harveyi* AY750578	99	Hong Kong, exterior
Hke12	UST040911-012	*Alteromonas macleodi* Y18228	98	Hong Kong, exterior
Hke6	UST040911-006	*Alteromonas macleodi* Y18228	99	Hong Kong, exterior
Hke5	UST040911-005	*Alteromonas marina* AF529060	98	Hong Kong, exterior
Hke4	UST040911-004	*Alteromonas macleodi* subsp. *fijiensi* AJ414399	98	Hong Kong, exterior
Hke3	UST040911-003	*Alteromonas marina* AF529060	99	Hong Kong, exterior
Hke9	UST040911-009	*Marinobacter aquaeolei* AJ000726	99	Hong Kong, exterior
Hke2	UST040911-002	*Psychrobacter* sp. ANT9171 AY167289	98	Hong Kong, exterior
Hki20	UST040911-035	*Vibrio tubiashi* X74725	97	Hong Kong, interior
Hki18	UST040911-033	*Vibrio natriegens* X74714	99	Hong Kong, interior
Hki17	UST040911-032	*Vibrio fischeri* AY292941	99	Hong Kong, interior
Hki16	UST040911-031	*Vibrio harveyi* AY750578	99	Hong Kong, interior
Hki15	UST040911-030	*Vibrio corallilyticus* AJ440004	96	Hong Kong, interior
Hki13	UST040911-028	*Vibrio harveyi* AY750578	99	Hong Kong, interior
Hki12	UST040911-027	*Vibrio harveyi* AY750578	99	Hong Kong, interior
Hki10	UST040911-025	*Vibrio corallilyticus* AJ440004	99	Hong Kong, interior
Hki8	UST040911-023	*Vibrio hollisae* AJ514911	96	Hong Kong, interior
Hki6	UST040911-021	*Vibrio corallilyticus* AJ440004	99	Hong Kong, interior
Hki4	UST040911-019	*Vibrio harveyi* AY264925	97	Hong Kong, interior
Hki2	UST040911-017	*Vibrio fischeri* AY292941	99	Hong Kong, interior
Hkr11	UST040911-046	*Alteromonas macleodii* Y18228	99	Hong Kong, substrata
Hkr10	UST040911-045	*Vibrio harveyi* X74693	98	Hong Kong, substrata
Hkr9	UST040911-044	*Alteromonas macleodii* AMY18228	99	Hong Kong, substrata
Hkr8	UST040911-043	*Alteromonas macleodii* Y18228	97	Hong Kong, substrata
Hkr6	UST040911-041	*Vibrio probioticus* AJ345063	95	Hong Kong, substrata
Hkr3	UST040911-038	*Vibrio aestuarianus* AJ845014	94	Hong Kong, substrata
α-Proteobacteria				
Cari307	UST030701-185	*Pseudovibrio denitrificans* AY486423	99	Bahamas, interior
Cari298	UST030701-181	*Pseudovibrio denitrificans* AY486423	99	Bahamas, interior
Carr515	UST030701-031	*Pseudovibrio denitrificans* AY486423	99	Bahamas, substrata
Carr504	UST030701-359	*Erythrobacter flavus* AF500005	98	Bahamas, substrata
Carr491	UST030701-344	*Erythrobacter flavus* strain SW-46 AF500004	99	Bahamas, substrata
Carr479	UST030701-331	*Erythrobacter flavus* strain SW-52 AF500005	100	Bahamas, substrata
Hki19	UST040911-034	*Ruegeria atlantica* D88527	96	Hong Kong, interior
Hki9	UST040911-024	*Silicibacter lacuscaerulensis* U77644	97	Hong Kong, interior
Firmicutes, Bacillales				
Care308	UST030701-186	*Bacillus hwajinpoensis* AF541966	98	Bahamas, exterior
Cari299	UST030701-182	*Bacillus megaterium* AF142677	98	Bahamas, interior
Hke11	UST040911-011	*Exiguobacterium* sp. AY744448	95	Hong Kong, exterior
Hke10	UST040911-010	*Exiguobacterium marinum* AY594266	97	Hong Kong, exterior
Hke 8	UST040911-008	*Exiguobacterium gaetbuli* AY594264	98	Hong Kong, exterior
Hke 7	UST040911-007	*Planococcus citreus* AF500008	98	Hong Kong, exterior
Hki7	UST040911-022	*Bacillus* sp. ROO40 AY188840	99	Hong Kong, interior
Hki3	UST040911-018	*Bacillus megaterium* AF142677	99	Hong Kong, interior
Hki1	UST040911-016	*Bacillus cereus* ZK CP000001	98	Hong Kong, interior
Hkr16	UST040911-050	*Bacillus thuringiensis* serovar *konkukian* AE017355	99	Hong Kong, substrata
Hkr15	UST040911-049	*Bacillus cereus* ZK CP000001	99	Hong Kong, substrata
Hkr14	UST040911-048	*Bacillus cereus* ATCC 14579 AE017013	97	Hong Kong, substrata
Hkr7	UST040911-042	*Exiguobacterium marinum* AY594266	98	Hong Kong, substrata
Hkr4	UST040911-039	*Bacillus megaterium* AJ717381	97	Hong Kong, substrata
Hkr2	UST040911-037	*Bacillus cereus* ATCC 14579 AE17013	99	Hong Kong, substrata
Hkr1	UST040911-036	*Bacillus cereus* ZK CP000001	99	Hong Kong, substrata
Bacteroides, Schingobacteria				
Carr492	UST030701-345	*Microscilla furvescens* AB078079	97	Bahamas, substrata
Actinobacteria, Actinobacteridae				
Hki14	UST040911-029	*Micrococcus luteus* AJ717367	99	Hong Kong, interior
Hki11	UST040911-026	*Micrococcus* sp. Ellin149 AF408991	99	Hong Kong, interior
Hki5	UST040911-020	*Micrococcus luteus* 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 *Callyspongia* sp.	*C. plicifera*	*Halichondria* sp.	*H. melanodocia*	Close match to the library	Probability %
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 trimethylsylyl 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-trimethylsiloxytoluene	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)	Extract *Callyspongia* sp.	*C. plicifera*	*Halichondria* sp.	*H. melanodocia*	Close match to the library	Probability %
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	Cyclodecasiloxane, 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			Tetrahydriaraucarolone	55
19.1		X			3-oxatricyclo[0.8.0.0(7,16)]triconta-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	Cyclodecasiloxane, eicosamethyl-	57
20.2	X		X		Unknown	<20
20.3	X				Unknown	<20
20.4	X		X		Unknown	<20
20.5	X				Tricycle[20.8.0.0(7,16)]triacontane, 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			Docosahexaenooic 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-dinorergost-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

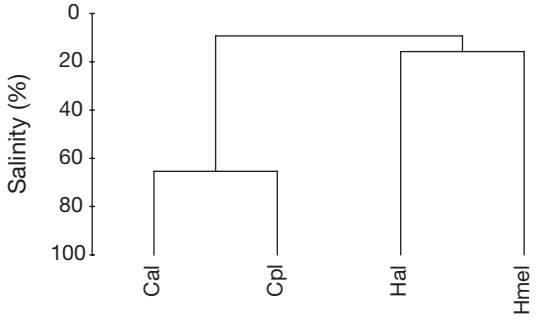

Fig. 2. Cluster analysis indicating similarities (%) among crude extracts of sponges *Callyspongia* sp. (Hong Kong, Cal), *C. plicifera* (the Bahamas, Cpl), *Halichondria* sp. (Hong Kong, Hal) and *H. melanodocia* (the Bahamas, Hmel)

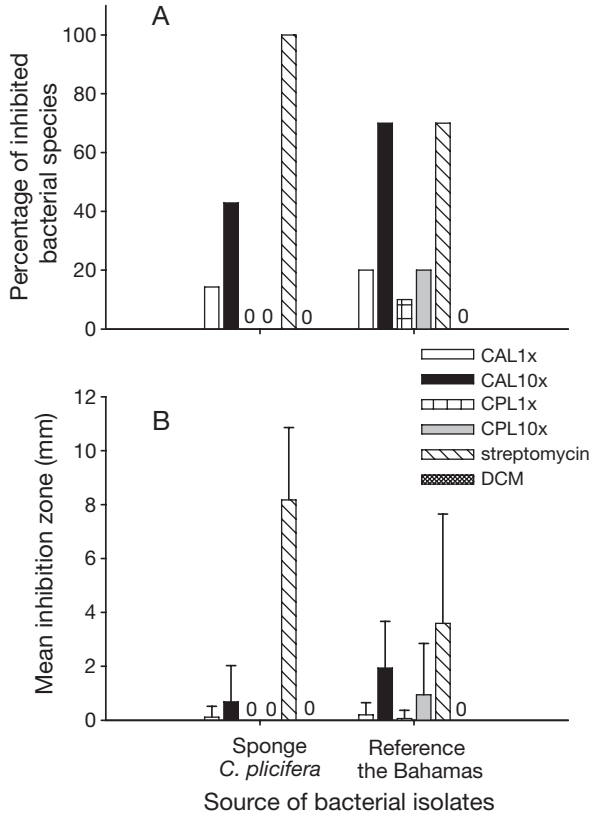

Fig. 3. Inhibition of growth of bacterial isolates from sponge *Callyspongia plicifera* and reference substrata from the Bahamas. In total, 9 isolates from reference substrata and strains from *C. plicifera* were tested. (A) Percentage of bacterial isolates inhibited by crude extracts of *Callyspongia* 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,

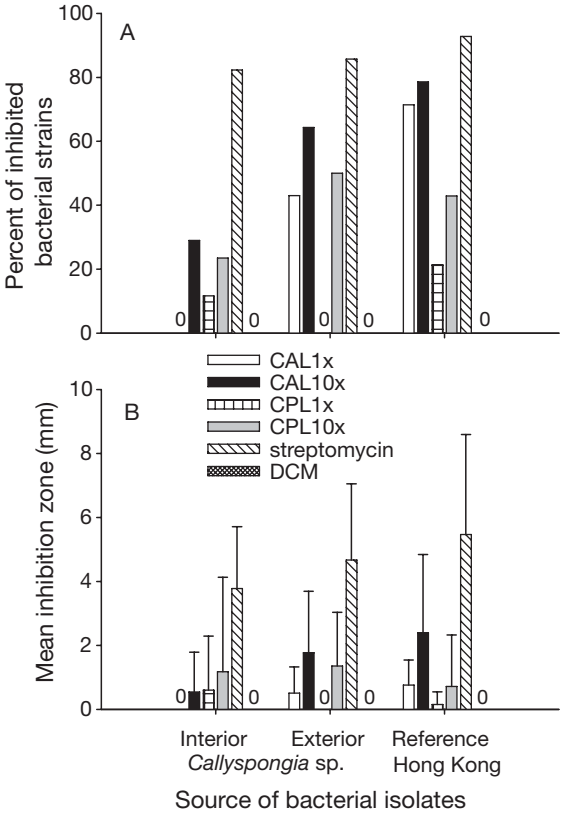

Fig. 4. Inhibition of growth of bacterial isolates from sponge *Callyspongia* sp. and reference substrata from Hong Kong. (A) Percentage of bacterial isolates inhibited by crude extracts of *Callyspongia* sp. (CAL) and *C. plicifera* (CPL) at TL (1×) and 10 times TL (10×) concentrations. (B) Mean ± SD 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.

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		

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 *Calyspongia plicifera* sponges 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 &

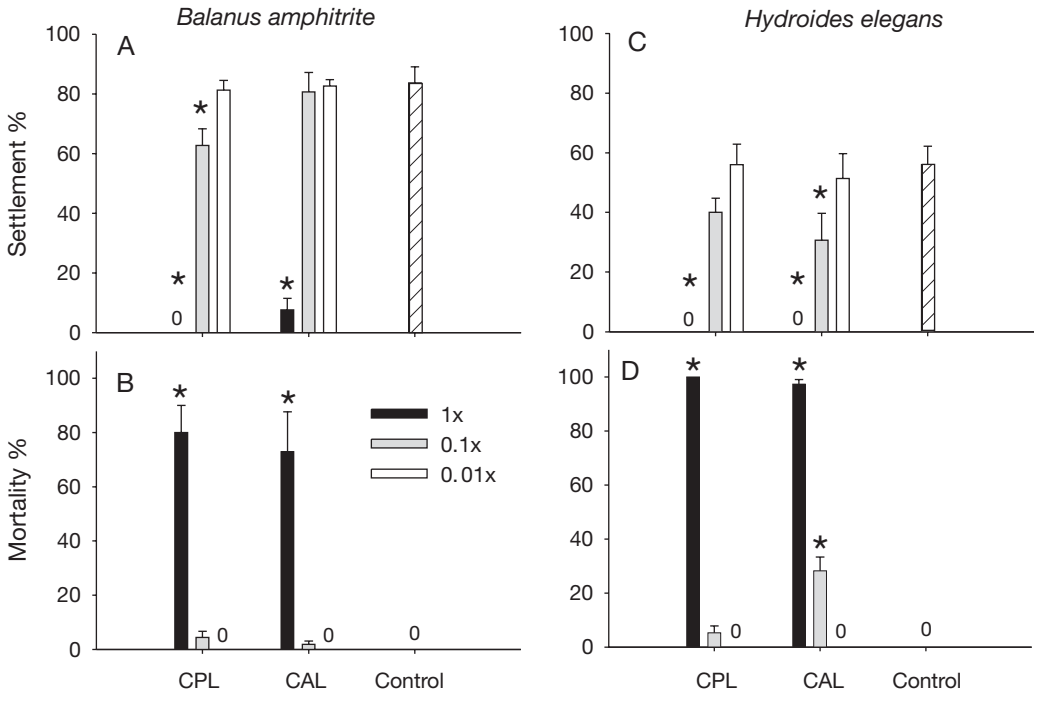

Fig. 5. (A, B) Barnacle *Balanus amphitrite* and (C, D) polychaete *Hydroides elegans* larval attachment and mortality in the presence of crude extracts of *Callyspongia* sp. from Hong Kong (CAL) and *C. plicifera* from the Bahamas (CPL) at TL (1×), 10-fold dilution (0.1×) and 100-fold dilution (0.01×). *Significantly different ($p < 0.05$) from the control (FSW with 3% DMSO) according to Dunnett's *t*-test. Results = mean ± 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 alkaloids (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 sceptrin 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 antifeeding 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 *Callispongiidae*, 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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LITERATURE CITED

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 1972:762–770

Amsler CH, Moeller CB, McClintock JB, Iken KB, Baker BJ (2000) Chemical defenses against diatom fouling in Antarctic marine sponges. Biofouling 16:29–45

Andersen RJ, van Soest RWM, Kong F (1996) 3-Alkylpiperidine alkaloids isolated from marine sponges in the order *Haplosclerida*. In: Pelletier SW (ed) Alkaloids: chemical and biological perspectives, Vol 10. Pergamon Press, Oxford, p 301–355

Assmann M, Lichte E, Pawlik JR, Koeck M (2000) Chemical defenses of the Caribbean sponges *Agelas wiedenmayeri* and *Agelas conifera*. Mar Ecol Prog Ser 207:255–262

Bakus GJ, Targett NM, Schulte B (1986) Chemical ecology of marine organisms: an overview. J Chem Ecol 12: 951–987

Beccerro MA, Uriz MJ, Turon X (1997) Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera Becilosclerida). Hydrobiologia 355:77–89

Beccero MA, Thacker RW, Turon X, Uriz MJ, Paul V (2003) Biogeography of sponge chemical ecology: comparisons of tropical and temperate defenses. Oecologia 135:91–101

Blackwood CB, Marsh T, Kim SH, Paul EA (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Appl Environ Microbiol 69:926–932

Blunt JW, Copp BR, Munro MHG, Northcote PT, Prinsep MR (2003) Marine natural products. Nat Prod Rep 20:1–131

Bryan JP, Kreider L, Qian PY (1997) Attachment of the polychaete *Hydroides elegans* on surfaces of the cheilostome bryozoan *Bugula neritina*: evidence for a chemically mediated relationship. J Exp Mar Biol Ecol 220:171–190

Buchan A, Newell SY, Butler M, Biers EJ, Hollibaugh JT, Moran MA (2003) Dynamics of bacterial and fungal communities on decaying salt marsh grass. Appl Envir Microbiol 69:6676–6687

Burja AM, Hill RT (2001) Microbial symbionts of the Australian Great Barrier Reef sponge, *Candidaspongia flabellata*. Hydrobiologia 461:41–47

Clarke KR, Warwick RM (1994) Changes in marine communities: an approach to statistical analysis and interpretation. Bourne Press, Bournemouth

Davis A (1998) Antifouling defence in a subtidal guild of temperate zone encrusting invertebrates. Biofouling 12: 305–320

Dobretsov S, Qian PY (2002) Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. Biofouling 18:217–228

Dobretsov S, Dahms HU, Qian PY (2004) Antilarval and antimicrobial activity of waterborne metabolites of the sponge *Callyspongia* (*Euplacella*) *pulvinata*: evidence of allelopathy. Mar Ecol Prog Ser 271:133–146

Dobretsov S, Dahms HU, Qian PY (2005a) Antibacterial and anti-diatom activity of Hong Kong sponges. Aquat Microb Ecol 38:191–201

Dobretsov S, Dahms HU, Tsoi MY, Qian PY (2005b) Chemical control of epibiosis by Hong Kong sponges: the effect of sponge extracts on micro- and macrofouling communities. Mar Ecol Prog Ser 297:119–129

Dunbar J, Ticknor LO, Kuske CR (2001) Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Envir Microbiol 67: 190–197

Eden PE, Schmidt TM, Blakemore RP, Pace NR (1991) Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rRNA-specific DNA. Int J Syst Bacteriol 41:324–325

Erpenbeck D, van Soest RWM (2005) A survey for biochemical synapomorphies to reveal phylogenetic relationships of halichondrid demosponges (Metazoa: Porifera). Biochem Syst Ecol 33:585–616

Faulkner DJ (2000) Marine natural products. Nat Prod Rep 17: 7–55

Fieseler L, Horn M, Wagner M, Hentschel U (2004) Discovery of the novel candidate phylum 'Poritobacteria' in marine sponges. Appl Environ Microb 70:3724–3732

Green G (1977) Ecology of toxicity in marine sponges. Mar Biol 40:207–215

Harder T, Lau SCK, Tam WY, Qian PY (2004) An ecologically realistic method to investigate chemically-mediated defense against microbial epibiosis in marine invertebrates by using TRFLP analysis and natural bacterial populations. FEMS Microbiol Ecol 47:93–99

Hentschel U, Schmid M, Wagner M, Fieseler L, Gernert C, Hacker J (2001) Isolation and phylogenetic analysis of bacteria with antimicrobial activities from Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. FEMS Microbiol Ecol 35:305–312

Hentschel U, Hopke J, Horn M, Friedrich A, Wagner M, Hacker J, Moore B (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. Appl Environ Microbiol 68:4431–4440

Hentschel U, Fieseler L, Wehrl M, Gernert C, Steinert M, Hacker J, Horn M (2003) Microbial diversity of marine sponges. Mueller WEG (ed) Marine molecular biotechnology. Springer Verlag, Berlin, p59–88

Hill RT (2004) Microbes from marine sponges: a treasure trove of biodiversity for natural product discovery. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM Press, Washington, DC, p 177–190

Kelly S, Jensen PR, Henkel TP, Fenical W, Pawlik JR (2003) Effects of Caribbean sponge extracts on bacterial attachment. Aquat Microb Ecol 31:175–182

Kelman D, Kashman Y, Rosenberg E, Ilan M, Ifrach I, Loya Y (2001) Antimicrobial activity of the reef sponge *Amphimedon viridis* from the Red Sea: evidence for selective toxicity. Aquat Microb Ecol 24:9–16

Lau SCK, Qian PY (1997) Phlorotannins and related compounds as larval settlement inhibitors of the tube-building polychaete *Hydroides elegans*. Mar Ecol Prog Ser 159: 217–227

Lau SCK, Mak KKW, Chen F, Qian PY (2002) Bioactivity of bacterial strains from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*. Mar Ecol Prog Ser 226:301–310

Lee OO, Qian PY (2003) Chemical control of bacterial epibiosis and larval attachment of *Hydroides elegans* in the Red Sponge *Mycale adhaerens*. Biofouling 19:171–180

Lee OO, Qian PY (2004) Potential control of bacterial epibiosis on the surface of the sponge *Mycale adhaerens*. Aquat Microb Ecol 34:11–21

Lee S, Malone C, Kemp PF (1993) Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar Ecol Prog Ser 101:193–201

Luna GM, Dell'Anno A, Danovaro M (2005) DNA extraction procedure: a critical issue for bacterial diversity assessment in marine sediments. Environ Microbiol 8:308–320

Montalvo NF, Mohamed NM, Enticknap JJ, Hill RT (2005) Novel actinobacteria from marine sponges. Antonie van Leeuwenhoek 87:29–36

Newbold RW, Jensen PR, Fenical W, Pawlik JR (1999) Antimicrobial activity of Caribbean sponge extracts. Aquat Microb Ecol 19:279–284

Olson JB, McCarthy PJ (2005) Associated bacterial communities of two deep-water sponges. Aquat Microb Ecol 39:47–55

Pechenik JA, Qian PY (1998) Onset and maintenance of metamorphic competence in the marine polychaete *Hydroides elegans* Haswell in response to three chemical cues. J Exp Mar Biol Ecol 226:51–74

Rittschof D, Clare AS, Gerhart DJ, Mary A, Bonaventura J (1992) Barnacle *in vitro* assays for biologically active substances: toxicity and attachment inhibition assays using mass cultured *Balanus amphitrite amphitrite* Darwin. Biofouling 6:115–122

Ruetzler K (1985) Associations between Caribbean sponges and photosynthetic organisms. In Ruetzler K (ed) New perspectives in sponge biology. Smithsonian Institution Press, Washington, DC, p 455–466

Santavy DL, Colwell RR (1990) Comparison of bacterial communities associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* and ambient seawater. Mar Ecol Prog Ser 67:73–82

Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). Biometrika 52:591–611

Sponga F, Cavaletti L, Lazzarini A, Borghi I, Ciciliato A, Losi D, Marinelli F (1999) Biodiversity and potentials of marine derived micro-organisms. J Biotechnol 70:65–69

Taylor MW, Schupp PJ, Dahlloef I, Kjelleberg S, Steinberg PD (2004) Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. Environ Microbiol 6:121–130

Taylor MW, Schupp PJ, de Nys R, Kjelleberg S, Steinberg PD (2005) Biogeography of bacteria associated with the marine sponge *Cymbastela concentrica.* Environ Microbiol 7:419–493

Thacker RW, Starnes S (2003) Host specificity of the symbiotic cyanobacterium *Oscillatoria spongeliae* in marine sponges, *Dysidea* spp. Mar Biol 142:643–648

Thakur NL, Hentschel U, Krasko A, Pabel CT, Anil AC, Müller WEG (2003) Antibacterial activity of the sponge *Suberites domuncula* and its primmorphs: potential basis for epibacterial chemical defense. Aquat Microb Ecol 31:77–83

Thiyagarajan V, Harder T, Qian PY (2002) Relationship between the energy reserve of cyprids and metamorphosis in the barnacle *Balanus amphitrite* Darwin (Cirripedia; Thoracica). J Exp Mar Biol Ecol 280:79–93

Thoms C, Horn M, Wagner M, Hentschel U, Proksch P (2003) Monitoring microbial diversity and natural product profiles of the sponge *Aplysina cavernicola* following transplantation. Mar Biol 142:685–692

Toth SI, Schmitz FJ (1994) Two cytotoxic peroxide-containing acids from the New Guinea sponge *Callyspongia* sp. J Nat Prod 57:123–127

Tsukamoto S, Kato H, Hirota H, Fusetani N (1997) Antifouling terpenes and steroids against barnacle larvae from marine sponges. Biofouling 11:283–291

van Soest RWM, Braekman JC (1999) Chemosystematics of Porifera: a review. Mem Qld Mus 44:569–589

Wang G, Kuramoto M, Uemura D (1996) Three novel antimicrofouling nitroalkyl pyridine alkaloids from Okinawan marine sponge *Callyspongia* sp. Tetrahedron Lett 37: 1813–1816

Webster NS, Hill RT (2001) The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha-Proteobacterium. Mar Biol 138:843–851

Zar JH (1999) Biostatistical analysis, 4th edn. Prentice Hall International, Upper Saddle River, NJ

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