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Surface bacterial community, fatty acid profile, and antifouling activity of two congeneric sponges from Hong Kong and the Bahamas

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ABSTRACT: Bacterial communities on the surfaces of 2 congeneric sponges, *Mycale adhaerens* from Hong Kong and *M. laxissima* from the Bahamas, were compared using conventional cultivation techniques and terminal restriction fragment length polymorphism (TRFLP) analysis — a culture-independent DNA fingerprinting technique. The bacterial community on the Hong Kong sponge was more diverse, in terms of the number and type of species isolated, and different from that on the Bahamas sponge, as evident from distinct clusters formed in TRFLP analysis. Distinctive bacterial types (i.e. TRFs) were commonly found on both sponge surfaces, but none of their bacterial isolates were common. At tissue level concentration, extract of the Hong Kong sponge inhibited the growth of a wide range of bacteria isolated from the Hong Kong reference surface, but did not affect any of those isolated from the Bahamas reference surface, indicating highly specific antibacterial activity. Extracts of both sponges, when incorporated into hydrogels and exposed to the natural environment for bacterial film development, dramatically altered the bacterial community in the films, either by shifting the bacterial composition or decreasing bacterial density. Settlement assays of the resulting films using larvae of the polychaete *Hydroides elegans* showed that the filmed hydrogels with Hong Kong sponge extracts either inhibited larval settlement or were toxic to the larvae; however, those with the Bahamas sponge extracts had no observable effect. HPLC (high performance liquid chromatography) and GC-MS (gas chromatography–mass spectrometry) analyses revealed different chemical profiles in the extracts; the Hong Kong sponge had a more diverse fatty acid profile. Our results suggest that the 2 congeneric sponges from geographically separated regions have 'species-specific', surface-associated bacterial communities and antifouling activities, which might be due to the differences in the chemical and fatty acid compositions of the 2 sponges. Alternately, different sponge-associated bacterial communities may reflect habitat differences in sympatric bacterial and fouling communities.

KEY WORDS: Congeneric sponges · Surface bacterial community · *Mycale* spp. · Antifouling · TRFLP · Fatty acid composition

INTRODUCTION

Marine sponges (Phylum: Porifera) are evolutionarily ancient metazoans and are well-known for their unique and diverse production metabolites showing various bioactivities (McClintock & Baker 2001, Paul et al. 2006). Numerous studies show potent antifeeding (Furrow et al. 2003), allelopathic (Engel & Pawlik 2000), antifungal (Tsukamoto et al. 1998), antibacterial (Thakur et al. 2004), anti-microfouling (Amsler et al. 2000), or anti-macrofouling (Kubanek et al. 2002) activities in different species of marine sponges. There is growing evidence that some of these bioactive metabolites are of microbial origin (Kon-ya et al. 1995), given the fact that sponges harbor a remarkable array of microorganisms, for instance, bacteria (Hentschel et

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al. 2001), cyanobacteria (Thacker & Starnes 2003), and fungi (Maldonado et al. 2005), both internally and on their surfaces. Through evolution, sponges may have developed a symbiotic relationship with certain kinds of microbes. Therefore, study of the microbial community associated with sponges is important for understanding sponge–microbe symbiosis, as well as the bioactivity displayed by sponges.

Different sponges from different locations and sponges of the same species collected at different times or of the same genus collected at the same location may possess highly consistent associations with certain kinds of bacteria (Margot et al. 2002, Lafi et al. 2005). These associated bacteria may co-evolve with sponges, forming stable, specific, and, perhaps, symbiotic interrelationships with their hosts (Hentschel et al. 2002). In contrast, 2 congeneric sponges *Callyspongia* spp. from different biogeographic regions have different bacterial associates (Qian et al. 2006). Therefore, it remains far from conclusive whether differences in sponge-associated bacterial communities can hold for other congeneric sponges from geographically separated locations a fact which could provide useful information on the co-evolution of bacteria and sponges.

Similar to the sponge–microbe association, bioactive compounds isolated from sponges may have evolved together with the associated microbes or in response to sympatric predators, competitors, or fouling organisms (McClintock & Baker 1997). Therefore, it is of great scientific interest to know if congeneric sponges from different locations produce similar metabolites or show similar bioactivity. In fact, there have been several studies attempting to answer this question, but their findings varied substantially. For instance, McClintock & Baker (1997) pointed out that the Antarctic congeners of *Haliclona* produced different types of secondary metabolites when compared with their tropical counterparts; in contrast, the Antarctic *Latrunculia* and *Dendrilla* produced similar chemicals in temperate and tropical congeners. Although Becerro et al. (2003) has recently suggested that chemical defenses against predators in tropical and temperate congeneric sponges are equally strong, there has been no study that compares the antifouling activity in congeneric sponges from tropical and sub-tropical regions.

The unique ability of sponges to adapt to any ecosystem can be due to the structural features of their membranes (Rod'kina 2005). The main components of sponge membranes, phospholipids, consist of unusual, long-chained fatty acids (Ando et al. 1998). Many unusual fatty acids isolated from sponges have potent cytotoxic (Tachibana et al. 1981), antimicrobial (Carballeira et al. 2002), and antifungal (Fusetani et al. 1993) activities. In addition, some branched fatty acids, particularly those with iso, anteiso, cyclopropyl, and monomethyl groups, are suggested to be of bacterial origin and specific to particular bacteria; because of this, they can be used as biological markers for bacterial associates in sponges (Gillian et al. 1988). Therefore, the study of fatty acid profiles in sponges may help understand the bacterial association, as well as the bioactivity, of sponges. However, the relationships of these 3 aspects in congeneric sponges have not been examined so far.

In the present study, we compared: (1) the surface-associated bacterial communities of the 2 congeneric sponges using a DNA fingerprinting technique, (2) the antifouling activity of the crude sponge extracts in 2 assays (inhibition of bacterial growth and attachment and inhibition of larval settlement of a major fouling polychaete *Hydroides elegans* in Hong Kong waters), and (3) the chemical and fatty acid compositions of the crude sponge extracts. We selected *Mycale adhaerens* from Hong Kong in the sub-tropical area and *M. laxissima* from the Bahamas in the tropical area for comparison. The genus *Mycale* was established in 1867 (Gray 1867), and there are >250 species in this genus at the time of writing. *M. adhaerens* Lambe was firstly discovered on the Pacific Coast of Canada and in the Bering Sea (Lambe 1893), can now be found in the San Juan Archipelago, Sea of Japan, and Greenland Sea, and is frequently associated with scallops. The surface-associated bacterial community and antifouling activity of this sponge have been recently studied by Qian's research group (Lee & Qian 2003, 2004, Lee et al. 2006), and its cytotoxic activity has been documented by Fusetani et al. (1991). On the other hand, *M. laxissima* Duhassaing & Michelotti are found in the Caribbean Sea (Duhassaing & Michelotti 1864) and along the Brazilian coastline; it is usually associated with hydrozoa and seaweed. So far, there have only been 2 studies on the cytotoxic and neurotoxic activities displayed by this sponge (Rangel et al. 2001, Freitas et al. 2003), but antifouling activity was not studied or compared.

MATERIALS AND METHODS

Collection of sponge tissues and bacteria. Tissue of the sponge *Mycale adhaerens* was obtained at a depth of 3 m from a fish farm in Long Harbour, Hong Kong (22° 27′ N, 114° 21′ E) in April 2003, while that of the sponge *M. laxissima* was obtained at a depth of about 5 m from Great Stirrup Cay, Bahaman Islands (25° 51′ N, 77° 52′ W) in July 2003. Sponges were carefully brought to the water surface and flushed with autoclaved 0.22 µm filtered seawater (AFSW) to remove loosely attached bacteria. Eight colonies of each sponge species, each with a surface area of

ca. 40 cm^2, were swabbed with sterile cotton tips to collect epibiotic bacteria; 4 of them were individually suspended in 1 ml of AFSW in 1.5 ml Eppendorf tubes for the isolation of epibiotic bacteria, while the other 4 were individually frozen in 0.8 ml of extraction buffer (100 mM of Tris-HCl, 100 mM of Na_2-EDTA, 100 mM of Na_2HPO_4, 1.5 M of NaCl, 1% of CTAB; at pH 8) for DNA fingerprinting analysis of bacterial communities. Reference bacterial communities were collected analogously from the surfaces of a polystyrene Petri dish (Falcon No. 1006) deployed in the close vicinity of the sponges for 7 d. For the chemical extraction of sponge tissue, colonies of approximately 550 ml of *M. adhaerens* and 440 ml of *M. laxissima* (determined by water displacement) were sealed in sterile plastic bags and transported back to the laboratory.

Isolation and identification of epibiotic bacteria. Isolation and identification of bacteria from the sponge and reference surfaces followed Lee & Qian (2003). Briefly, bacterial suspensions, after diluting with AFSW to 10- and 100-fold, were spread on nutrient agar (0.3% yeast extract, 0.5% peptone, 1.5% agar in AFSW) and incubated at 28°C under a 15 h light:9 h dark photoperiod for 48 h. After incubation, morphologically distinct colonies were isolated and identified by comparative analysis of 16S rRNA gene sequences. The primers used were 355F and 1055R, and the PCR (polymerase chain reaction) conditions were the same as described in Lee & Qian (2003). Sequences from individual primers were assembled using the Sequencher software package (Gene Codes), and the assembled nucleotide sequences were compared with those deposited in GenBank (www.ncbi.nlm.nih.gov) to obtain the closest match.

DNA fingerprinting analysis of bacterial communities. The extraction and purification of total bacterial DNA from the samples followed the SDS (sodium dodecyl sulfate)-based method described in Liu et al. (1997). For the analysis of bacterial communities by terminal restriction fragment length polymorphism (TRFLP) analysis, the 16S rRNA genes in DNA extracts were amplified by PCR using the primers 341F and 926R-Fam, following the conditions stated in Lee & Qian (2004). PCR products were cleaved with 10 U of the restriction enzyme *Msp*I at 37°C for 6 h, followed by purification with the Wizard® PCR preps DNA purification system (Promega) according to the manufacturer's protocol. Then, 10 µl of purified products mixed with 0.5 µl of internal size standard (ET-550R, Amersham) was denatured at 95°C for 2 min, snap cooled on ice, and analyzed by electrophoresis on a MegaBACE genetic analyzer (Amersham) operated in the genotyping mode. After electrophoresis, the size of the fluorescently labeled terminal restriction fragments (TRFs) was determined by comparison with the internal standard using the software Fragment Profiler (Amersham).

Extraction of sponge tissue. Sponge tissues for chemical extraction were blot dried, cut into small pieces, and extracted twice in equal volumes of a 1:1 mixture of methanol/chloroform (MeOH/$CHCl_3$) for 12 h each, with gentle agitation. After extraction, sponge tissue was removed from the solvents by centrifugation. Then, the organic extract was separated into 2 layers (MeOH and $CHCl_3$) in a separation funnel, and each layer was dried by rotary evaporation. The dry MeOH and $CHCl_3$ extracts were re-dissolved in one-tenth volume of the original sponge tissue of autoclaved double-distilled water (ddH_2O) and $CHCl_3$, respectively, generating extracts at 10× tissue-level concentration (TLC). TLC is measured on a volumetric basis assuming that compounds extracted from a specimen are homogenous distributed over the whole tissue volume. Without knowing the actual distribution of compounds in tissues, TLC is more relevant and acceptable measurement in ecological studies. In total, 14.3 and 10.4 g (dry weight) of MeOH extracts and 10.1 and 4.6 g of $CHCl_3$ extracts were obtained from 550 ml of *Mycale adhaerens* and 440 ml of *M. laxissima*, respectively.

Disc-diffusion assays. Antibacterial growth activity of the sponge tissue extracts were assessed using disc-diffusion assays. Bacterial isolates from the reference surfaces were grown to the stationary phase in nutrient broth. Then, 200 µl volumes of these cultures were individually spread on nutrient agars. The sponge tissue extracts were diluted to 1× TLC with corresponding solvents. Sterile paper discs (6.5 mm diameter, Whatman No. 1), each loaded with 20 µl of the extracts (equivalent to the volume of a paper disc) were subsequently placed onto the inoculated agar, with replications (n = 2). Pure solvents and streptomycin (50 µg $disc^{-1}$) served as controls. After 24 h of incubation at 28°C, the width of the growth inhibition zone measured from the edge of the paper disc to the unaffected bacterial lawn was recorded.

Phytagel assays. Antibacterial attachment activity of sponge tissue extracts were assessed using phytagel assays as described in Harder et al. (2004) with slight modifications. Briefly, 3 ml of aliquots of MeOH or $CHCl_3$ extract at 20× TLC was transferred to individual 50 ml sterile polypropylene tubes (Falcon 2096), each of which contained 27 ml of 4% (w/v) hydrogel (Phytagel, Sigma Chemical) solution prepared in hot ddH_2O (ca. 70°C). After cooling to room temperature to yield transparent gel cylinders containing extracts at a final concentration of 2× TLC and keeping overnight at 4°C, the gel cylinders were sliced into 5 mm thick gel discs (volume of each disc is approximately 2.5 ml) using sterile razor blades. Crude extract at a final

extract concentration of 2× TLC was used, in considering the diffusion rate of compounds from the gel discs, to ensure compounds are present throughout the assays. Positive control gel discs were prepared accordingly using a concentrated antibiotic solution, which yielded a final concentration of 219 mg l^{-1} penicillin + 365 mg l^{-1} streptomycin in the gel cylinder, and negative control gel discs were made with pure ddH_2O or $CHCl_3$ accordingly. Twenty replicates were prepared for each treatment.

Gel discs were pierced with fishing line and anchored 2 m below the water surface at the pier of the Hong Kong University of Science and Technology. After 72 h exposure to natural flowing seawater, the gel discs were retrieved and rinsed with AFSW; 12 of which (4 replicates) were subject to extraction of bacterial community DNA and TRFLP analysis as mentioned above, 5 were directly used for larval settlement bioassays as follows, and the remaining 3 were fixed with 4% formaldehyde, stained with DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 µg ml^{-1} for 10 min, and visualized at 1000× magnification in 10 randomly chosen fields of view under an epifluorescent microscope to determine the bacterial cell density.

Larval settlement bioassays. Larvae of *Hydroides elegans* were obtained and raised according to Lee & Qian (2003). Five gel discs directly retrieved from the field (i.e. coated with natural biofilms) were placed in polystyrene Petri dishes (Falcon No. 1006) and covered with 7 ml of AFSW. To investigate the effect of biofilms on larval settlement, 20 competent larvae were added to each Petri dish that contained a biofilm-coated gel discs. After 24 h of incubation at 24°C on a 15 h light: 9 h dark photoperiod, the percentage of larvae that had undergone settlement was determined by counting the number of individuals that had adhered to the gel surface and produce calcified tubes and tentacles. Additionally, percent survivorship in each treatment was scored as the sum of settled and free-swimming larvae. Bioassays to investigate the potential larval settlement inhibition of sponge extracts in gel discs followed the same procedures, except that gel discs with prior exposure to seawater but with the biofilms being removed using cotton tips for TRFLP analysis were placed in individual wells and that each well received 20 larvae that had been exposed to 10^{-4} M IBMX (3-isobutyl-1-methylxanthine) 30 min prior to the bioassays. IBMX is a pharmacological compound that can effectively induce larval settlement of *H. elegans*.

Determination of chemical profile and fatty acid composition of sponge extracts. Crude MeOH and $CHCl_3$ extracts of *Mycale adhaerens* and *M. laxissima* at 20× TLC were analyzed using the reverse phase HPLC (Lichrospher 100 RP C18 EC 5, 250 × 4 mm i.d.; gradient 5% aqueous CH_3CN to 85% CH_3CN) at a flow rate of 1 ml min^{-1}.

Crude $CHCl_3$ extracts of the 2 sponges at 1× TLC and methyl esters of fatty acids (MEFAs) of total lipids in the extracts were analyzed using coupled gas chromatography–mass spectrometry (GC-MS). Total lipids were obtained by dissolving the extracts in $CHCl_3$:CH_3OH:ddH_2O (1:1:0.9). After phase separation at 4°C, the organic phase was collected and evaporated to dryness *in vacuo*. Fatty acids in the crude lipid samples (5 mg) were transmethylated according to Carreau & Dubacq (1978) and then dissolved in 0.5 ml of ddH_2O. MEFAs were extracted with 2 ml of hexane 3 times, and the organic phase was combined and evaporated to dryness *in vacuo*. MEFAs were purified by thin layer chromatography using benzene as solvent and analyzed by GC-MS. GC was performed on relatively nonpolar capillary columns (CP-Sil 8 CB-MS, 30 m length, 0.25 µm film thickness, 0.25 mm i.d.; Varian 3800). For the crude extracts, temperature gradients used were initially from 65 to 120°C at 10°C min^{-1} and then 120 to 310°C at 12°C min^{-1}, with subsequent constant temperature at 310°C for 10 min. For MEFAs of the 2 extracts, temperature gradients used were initially from 180 to 220°C at 3°C min^{-1} and then 220 to 260°C at 2°C min^{-1}, with subsequent constant temperature at 260°C for 5 min. Helium served as the carrier gas. MS was obtained by electron impact ionization at 70 eV (Saturn 200 ion-trap). Each peak shown in a chromatogram represents an individual compound/fatty acid in the extracts and was identified by comparison with the NIST GC-MS library and authentic standards of 15:0, 16:0, and 18:0.

Statistical analysis. For TRFLP analysis, TRFs that were <50 fluorescence units in intensity, <35 bp in size, or >500 bp in size were excluded from statistical analysis, in order to screen off background noise, to avoid pseudo-TRFs derived from primers, and to avoid inaccurate size determination, respectively. TRFLP profiles were analyzed using multivariate techniques. The Bray-Curtis coefficient was calculated based on the total number of TRFs in all samples and the presence or absence of these TRFs in individual samples to produce similarity matrices. Due to differential amplification of DNA during PCR, the signal intensity of TRFs may not be an accurate reflection of the actual abundance of the corresponding bacterial types and, thus, was not considered in the analysis. Similarity matrices were utilized for agglomerative hierarchical clustering, using the PRIMER program (Plymouth Routines In Multivariate Ecological Research; Clarke & Warwick 1994) to create dendrograms showing the similarity among samples. For the larval settlement bioassays, data in the form of percentages of larval survivorship and settlement were arcsine-transformed before statis-

tical analysis. Normality of data was checked with Shapiro-Wilk's test, and homogeneity of variance within samples was analyzed by Cochran's test. Data that met the assumptions of parametric tests were analyzed using Student's *t*-test, while data that did not meet the assumptions of parametric analysis were analyzed by Mann-Whitney *U*-test.

RESULTS

Isolation and identification of epibiotic bacteria

Altogether 20 and 11 morphologically distinct bacteria were isolated from the surfaces of *Mycale adhaerens* and *M. laxissima*, respectively (these bacteria are referred to as 'HK sponge isolates' and 'Bahamas sponge isolates', respectively, hereafter). Comparative sequence analysis of the 16S rRNA gene revealed that 11 HK sponge isolates belonged to the γ-subdivision of *Proteobacteria*, 3 to the α-subdivision of *Proteobacteria*, 5 to the division of Gram-positive, and 1 to the *Cytophaga–Flexibacter–Bacteroidetes* division (Table 1, present study; Lee & Qian 2003). Of the 11 Bahamas sponge isolates, 4 belonged to the γ-subdivision of *Proteobacteria*, 5 to the α-subdivision of *Proteobacteria*, and 2 to the division of Gram-positive (Table 1). On the other hand, 36 and 24 bacteria were isolated from the reference surfaces (i.e. polystyrene Petri dish deployed in the close vicinity of the sponges for 7 d) in Hong Kong and the Bahamas, respectively (referred to as 'HK reference isolate' and 'Bahamas reference isolate' hereafter). Among the 36 HK reference isolates, 24 belonged to the γ-subdivision of *Proteobacteria*, 2 to the α-subdivision of *Proteobacteria*, 8 to the division of Gram-positive, and 2 to the *Cytophaga–Flexibacter–Bacteroidetes* division (Table 2, present study; Lee & Qian 2003). The Bahamas reference isolates could also be affiliated with the γ-subdivision of *Proteobacteria* (15 isolates), the α-subdivision of *Proteobacteria* (3 isolates), the division of Gram-positive (3 isolates), and the *Cytophaga–Flexibacter–Bacteroidetes* division (3 isolates). Results indicated that no bacterium was found in common on surfaces of either the sponge or reference isolates. The culturable bacterial community on the surface of *M. adhaerens* was more diverse than that on *M. laxissima*, but less diverse than that on the reference surfaces from either location in terms of the total number of isolates and the number of genera affiliated.

Table 1. *Mycale* spp. Phylogenetic affiliations of bacteria isolated from surface of sponges from Hong Kong and the Bahamas. Bacteria were isolated from 4 different sponge colonies for each species. Isolates from *M. adhaerens* and *M. laxissima* were annotated with prefix 'A' and 'L', respectively. The 16S rRNA gene sequences of individual bacterial isolates were compared to the nucleotide sequences deposited in GenBank. Closest phylogenetic affiliation for each isolate is indicated by strain name, accession number, and similarity

Isolate	Closest phylogenetic affiliation: Strain	Accession No.	Similarity (%)
γ-*Proteobacteria*			
M. adhaerens (Hong Kong)			
A5	*Alteromonas alvinellae*	AF288360	99
A10	*Alteromonas alvinellae*	AF288360	98
A12	*Microbulbifer hydrolyticus*	U58338	97
A20	*Pseudoalteromonas piscicida*	AF297959	97
A6	*Pseudoalteromonas* sp. S9	AB013442	98
A13	*Shewanella algae*	X81621	98
A1	*Vibrio nereis* ATCC 25917T	X74716	96
A2	*Vibrio halioticoli*	AB000392	97
A9	*Vibrio furnissii* ATCC 35016T	X76336	96
A11	*Vibrio fluvialis* NCTC 11327T	X76335	97
A14	*Vibrio* sp. BV25Ex	AF319769	99
M. laxissima (Bahamas)			
L2	*Microbulbifer cystodytense*	AJ620879	100
L5	*Pelagiobacter variabilis*	AB167354	98
L10	*Pelagiobacter variabilis*	AB167354	99
L11	*Vibrio harveyi* OVL 99-52331-A	AY264926	98
α-*Proteobacteria*			
M. adhaerens (Hong Kong)			
A7	*Alpha proteobacterium* ISHR1	AB013442	99
A8	Uncultured *Ruegeria Ctax-Med-2*	AF259604	98
A15	*Alpha proteobacterium* MBIC1876	AB026194	99
M. laxissima (Bahamas)			
L4	*Pseudovibrio denitrificans* DN34	AY486423	99
L6	*Pseudovibrio denitrificans* DN34	AY486423	99
L7	*Pseudovibrio denitrificans* DN34	AY486423	99
L12	*Pseudovibrio denitrificans* DN34	AY486423	99
L3	*Ruegeria* sp. AS-36	AJ391197	98
Cytophaga–Flexibacter–Bacteroidetes			
M. adhaerens (Hong Kong)			
A3	*Tenacibaculum mesophilum* MBIC1543	AB032502	97
Gram-positive			
M. adhaerens (Hong Kong)			
A4	*Bacillus licheniformis* strain B	AF276309	99
A17	*Kocuria rhizophila* JPL-9	AY030315	98
A16	*Micrococcus kristinae*	X80749	99
A18	*Micrococcus* sp. Wuba57	AF336358	99
A19	*Staphylococcus cohnii*	AB009936	99
M. laxissima (Bahamas)			
L1	*Bacillus vietnamensis*	AB099708	96
L8	*Janibacter brevis*	AJ310085	99

TRFLP analysis of bacterial communities

Bacterial communities from different types of surfaces (sponge and reference) displayed distinctive TRFs (Table 3). For instance, TRFs of 179, 310, 315, and 373 bp were observed on 1 type of surface only: *Mycale laxissima*, *M. adhaerens*, the Hong Kong reference, and the Bahamas reference, respectively. A TRF of 247 bp was found exclusively on the sponge surfaces from 2 geographically separated locations, but not on either of the reference surfaces, while TRFs of 248 and 302 bp were found on both of the sponge surfaces regardless of their presence on the reference surfaces (Table 3). On the other hand, TRFs of 182, 300, and 314 bp were observed exclusively on the reference surfaces from the 2 locations, but not on any of the sponge surfaces. The number of discernible TRFs derived from each of the sponge- and reference-surface bacterial communities ranged from 8 to 16, with those from the Bahamas reference surfaces displaying the highest number of TRFs, while those from the sponge surfaces at the same location showed the lowest. Cluster analysis of TRF patterns indicated that replicated samples from each type of surfaces formed distinct clusters with >60% similarity among replicates (Fig. 1). Samples from the surfaces of *M. adhaerens* and *M. laxissima* formed another cluster, which was distantly related to the cluster formed by the samples from the reference surfaces at the 2 locations (similarity ~15%; Fig. 1).

Antibacterial growth activity of extracts from *Mycale* spp.

All HK reference isolates, except 2 (009 and 011), and all Bahamas reference isolates, except 3 (479, 484, 491), were susceptible to the streptomycin control (50 µg disc^{-1}) (Table 4). Of the 36 HK reference isolates, 18 (i.e. 50%) were susceptible to the $CHCl_3$ extract of *Mycale adhaerens* from Hong

Table 2. Phylogenetic affiliations of bacteria isolated from surface of polystyrene Petri dish deployed in close vicinity of the sponges for 7 d (reference) in Hong Kong and the Bahamas (n = 4). The 16S rRNA gene sequences of individual bacterial isolates were compared to the nucleotide sequences deposited in GenBank. Closest phylogenetic affiliation for each isolate is indicated by strain name, accession number, and similarity

Isolate	Closest phylogenetic affiliation: Strain	Accession No.	Similarity (%)
γ-*Proteobacteria*			
Hong Kong reference			
030	*Alteromonas* sp. KT0903	AF235119	99
020	Endocytic bacterium Noc15	AF262741	99
039	Gram-negative bacterium CKT1	AB036070	98
040	Gram-negative bacterium CKT1	AB036070	96
003	*Idiomarina loihiensis*	AF288370	96
026	Marine bacterium Tw-3	AY028198	97
012	North Sea bacterium H7	AF069665	98
014	*Photobacterium phospheum*	Z19107	98
016	*Photobacterium* sp. KT0248	AF235127	96
028	*Pseudoalteromonas haloplanktis* subsp. *Tetradonis*	AF214730	99
029	*Pseudoalteromonas haloplanktis* subsp. *Tetradonis*	AF214730	99
019	*Pseudoalteromonas issachenkonii* KMM3549	AF316144	99
007	*Pseudoalteromonas* sp. AS-27	AJ391188	99
006	*Pseudoatleromonas* sp. UST991130-004	AF465392	98
004	*Shewanella* sp. GIT-33	AF249336	98
010	*Vibrio harveyi* M4	AY046956	97
008	*Vibrio rumoence*	AB013297	98
015	*Vibrio splendidus* B17	AY046955	98
023	*Vibrio* sp. ED4	AY035895	99
037	*Vibrio* sp. EN276	AB038023	98
033	*Vibrio* sp. UST991130-040	AF465358	98
034	*Vibrio* sp. UST991130-040	AF465358	98
024	*Vibrio scophthalmi*	U46579	98
032	*Vibrio splendidus* B17	AY046955	99
Bahamas reference			
473	*Alteromonas macleodii* DSM 6062	AMY18228	98
474	*Alteromonas macleodii* subsp. *Fijiensi*	AL414399	97
483	*Alteromonas macleodii* subsp. *Fijiensi*	AJ414399	98
501	*Alteromonas macleodii* subsp. *Fijiensi*	AJ414399	96
480	*Alteromonas marina* SW-47	AF529060	98
484	*Alteromonas marina* SW-47	AF529060	96
489	*Alteromonas marina* SW-47	AF529060	98
507	*Alteromonas marina* SW-47	AF529060	99
476	*Alteromonas* sp. UST020129-020	AY241417	93
506	*Alteromonas* sp. UST010723-005	AY241400	97
503	*Marinobacter aquaeolei*	AJ000726	100
493	*Pelagiobacter variabilis*	AB167354	99
519	*Pseudoalteromonas flavipulchra* A	F297958	99
495	*Pseudoalteromonas* sp. An2	AJ551143	98
516	*Pseudoalteromonas* sp. S9	U80834	98
α-*Proteobacteria*			
Hong Kong reference			
035	Marine *alpha proteobacterium* AS-19	AJ391181	97
013	Uncultured marine eubacterium HstpL28	AF159650	97
Bahamas reference			
479	*Erythrobacter flavus* SW-52	AF500005	100
491	*Erythrobacter flavus* SW-52	AF500004	99
500	*Erythrobacter flavus* SW-52	AF500005	97

(Table continued on facing page)

Table 2 (continued)

Isolate	Closest phylogenetic affiliation		
	Strain	Accession No.	Similarity (%)
Cytophaga–Flexibacter–Bacteroidetes			
Hong Kong reference			
001	*Tenacibaculum mesophilum* MBIC4357	AB032504	98
011	*Flavobacteirum salegens*	M92279	97
Bahamas reference			
487	*Cytophaga* sp. J18-M01	AB017046	91
492	*Fabibacter halotolerans*	DQ080995	100
499	*Roseivirga spongocola*	DQ080996	100
Gram-positive			
Hong Kong reference			
025	*Bacillus benzoevorans*	AY043085	97
009	*Bacillus clausii* LMG19634	AF329475	98
017	*Bacillus* sp. 171544	AF071856	99
022	*Bacillus* sp. KL-152	AY030333	96
005	*Bacillus* sp. OS-5	AJ296095	97
002	*Exiguobacterium acetylicum*	D55730	97
018	*Microbacterium esteraromaticum*	Y17231	99
031	*Planococcus citreus*	AF237975	98
Bahamas reference			
481	*Micrococcus luteus*	AJ409096	99
496	*Bacillus hwajinpoensis*	AF541966	97
512	*Halobacillus karajiensis* DSM 14948	AJ486874	98

Kong, but none was affected by the MeOH extract of the sponge from the same location (Table 4). Similarly, no Bahamas reference isolate was susceptible to the MeOH extract of *M. laxissima* from the Bahamas, but weak inhibition by the $CHCl_3$ extract of the sponge on the growth of 1 isolate from the same location was observed. When sponge extracts from one location were tested against the reference bacteria isolated from the other location, different levels of antibacterial growth activity were observed; MeOH and $CHCl_3$ extracts of *M. laxissima* inhibited the growth of 7 (i.e. 19.4%) and 5 (i.e. 13.9%) of the HK reference isolates, respectively, while extracts from *M. adhaerens* did not affect the growth of any Bahamas reference isolates. When extracts from different sponges were tested against the growth of bacteria isolated from the sponge surfaces, only 1 epibiotic bacterium from *M. adhaerens* was susceptible (Table 4).

Antibacterial attachment activity of extracts from *Mycale* spp.

After 72 h of exposure to flow-through seawater in the natural environment, bacterial densities on the surfaces of hydrogels prepared with ddH_2O alone and control hydrogels treated with $CHCl_3$ alone were ~10^5 cells mm^{-2}, whereas the hydrogels incorporated with antibiotics had a significantly lower bacterial cell density of 10^4 cells mm^{-2} when compared with the ddH_2O control (Mann-Whitney *U*-test; p = 0; Fig. 2a). Bacterial density on hydrogels treated with $CHCl_3$ extract of *Mycale adhaerens* was similar to that on the ddH_2O control hydrogels (Mann-Whitney *U*-test; p = 0.08). In contrast, bacterial density on hydrogels treated with MeOH extract of *M. adhaerens* was 4×10^4 cells mm^{-2}, which was significantly lower than in the ddH_2O control (Mann-Whitney *U*-test; p = 0), whereas bacterial densities on hydrogels treated with extracts of *M. laxissima* ranged from 1.8×10^5 to 2.2×10^5 cells mm^{-2}, which was significantly higher than in the ddH_2O and $CHCl_3$ controls (Mann-Whitney *U*-test; p = 0).

Bacterial communities on the ddH_2O and $CHCl_3$ controls were highly similar, as indicated by the occurrence of 18 TRFs in common (Table 5) and >60% similarity in the cluster analysis (Fig. 3), whereas those on the antibiotic control were substantially different from the ddH_2O and $CHCl_3$ controls in terms of the length and average number of TRFs. Bacterial communities on the hydrogels incorporated with different extracts of *Mycale adhaerens* and *M. laxissima* generally displayed high dissimilarity among themselves, as well as compared to controls (Fig. 3, Table 5). Four TRFs (91, 129, 182, and 314 bp) were excluded from the bacterial communities developed on all different kinds of extract-incorporated hydrogels when compared with the ddH_2O and $CHCl_3$ controls, while some TRFs (e.g. 368, 370, and 422 bp) were absent from the bacterial communities developed on certain kinds of extract-incorporated hydrogels (Table 5). Several other TRFs (e.g. 42, 81, 127, and 301 bp) were found exclusively on the extract-incorporated hydrogels, but not on any of the ddH_2O and $CHCl_3$ controls (Table 5). Cluster analysis also indicated that the bacterial communities that developed on all different kinds of extract-containing hydrogels and on the antibiotic control differed substantially from those on the ddH_2O and $CHCl_3$ controls (Fig. 3). Bacterial communities on the hydrogels incorporated with extracts of *M. adhaerens* formed a cluster, which was distantly related to the cluster formed by the antibiotic control and the $CHCl_3$ extract of *M. laxissima*.

Table 3. *Mycale* spp. Terminal restriction fragments (TRF) observed in bacterial communities from surfaces of 4 ind. of sponges *M. adhaerens* (HKS) and *M. laxissima* (BS) in comparison to those from reference surfaces of Hong Kong (HKR) and the Bahamas (BR). Data are number of occurrences of a particular TRF among 4 replicates. No data: total absence of TRF in a sample

Length of TRF (bp)	HKS	BS	HKR	BR
38				4
40				3
41, 71				4
74				2
75				4
76				3
86		4		
88			4	
128			2	
149	2			
179, 180		4		
182			3	3
216				3
247	3	4		
248	4	4		4
249	2			
299		4		
300			4	4
301		2	4	4
302	2	2		4
308		3		
309		4		
310, 311	4			
312				4
314			4	3
315			4	
366			3	
368			4	
369	3		2	
370			4	
371	3			
372	2			
373				4
374				2
375	3		4	
419, 420	2			
422	4		4	
423	2			4
Average (±SD) no. of TRFs	10.3 ± 1.7	8.8 ± 1.3	11.5 ± 1.3	15.8 ± 1.5

Antilarval settlement activity of extracts from *Mycale* spp.

After 24 h of exposure to the filmed hydrogels, 100% survivorship was recorded for larvae of *Hydroides elegans* that were exposed to all different kinds of hydrogels, except those incorporated with the $CHCl_3$ extract of *Mycale adhaerens*, which caused >50% reduction in percent survivorship.

Larval settlement on the filmed hydrogels incorporated with MeOH and $CHCl_3$ extracts of *Mycale laxissima* were ~80%, which was not significantly different from those on the filmed hydrogels of the ddH_2O and $CHCl_3$ controls (Mann-Whitney *U*-test; $p > 0.31$; Fig. 2b). Percent larval settlement on the antibiotic control and on the filmed hydrogels treated with MeOH and $CHCl_3$ extracts of *M. adhaerens* ranged from 34 to 53%, which was significantly lower than that on the ddH_2O and $CHCl_3$ controls (Mann-Whitney *U*-test; $p = 0.08$). Larval settlement on the hydrogels that had their biofilms removed was not significantly different across all treatments (data not shown).

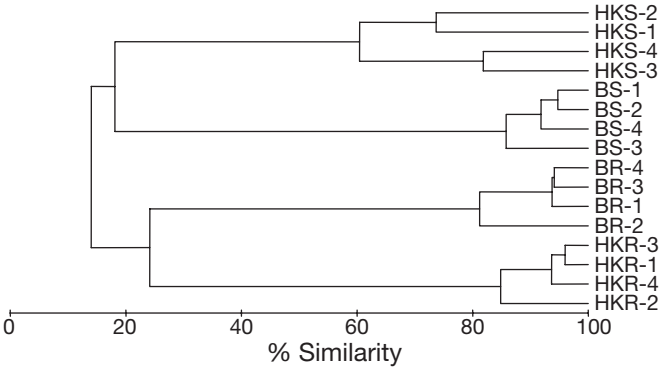

Fig. 1. *Mycale* spp. Dendrogram showing similarity of bacterial communities on the surfaces of 4 ind. of sponges *M. adhaerens* (HKS-1 to HKS-4) and *M. laxissima* (BS-1 to BS-4) in comparison with those on reference surfaces from Hong Kong (HKR-1 to HKR-4) and the Bahamas (BR-1 to BR-4)

Table 4. *Mycale* spp. Antibacterial activity of extracts from *M. adhaerens* and *M. laxissima* on growth of bacteria isolated from reference and sponge surfaces. Crude extracts were obtained by combining extracts from >1 sponge for each species and tested at 1× tissue-level concentration. Data are number of isolates susceptible to the extracts. Numbers in parentheses: total number of bacterial isolates from that particular type of surface

Paper disc loaded with	Test against growth of:			
	Hong Kong reference isolates (36)	Bahamas reference isolates (24)	*M. adhaerens* isolates (20)	*M. laxissima* isolates (11)
50 µg Streptomycin (positive control)	34	21	18	10
ddH_2O (negative control)	0	0	0	0
$CHCl_3$ (negative control)	0	0	0	0
MeOH extract from *M. adhaerens*	0	0	0	0
$CHCl_3$ extract from *M. adhaerens*	18	0	1	0
MeOH extract from *M. laxissima*	7	0	0	0
$CHCl_3$ extract from *M. laxissima*	5	1	0	0

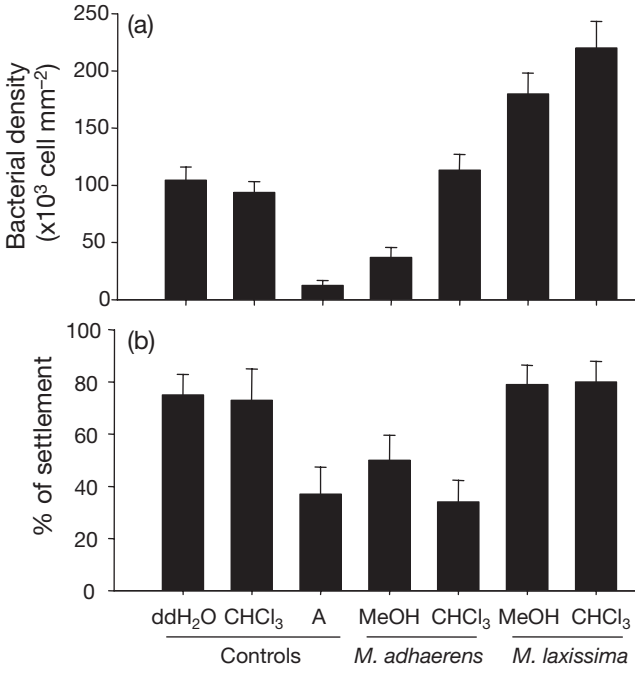

Fig. 2. *Hydroides elegans*, *Mycale* spp. (a) Bacterial density and (b) percent settlement of *H. elegans* larvae on surfaces of hydrogels with extracts from tissues of *M. adhaerens* and *M. laxissima* at 2× tissue-level concentration. Crude extracts were obtained by combining extracts from >1 sponge for each species. 'ddH$_2$O', 'CHCl$_3$' and 'A' represent the 3 controls: hydrogels made with ddH$_2$O, hydrogels incorporated with CHCl$_3$ solvent, and hydrogels incorporated with antibiotics, respectively. Hydrogels had been exposed to flow-through seawater in the natural environment for 72 h. Data for bacterial density are mean values (+SD) of triplicate measurements, with pseudo-replication (n = 10) in each replicate, while data for percent larval settlement are expressed as mean (+SD for 5 replicates) percent of total number of larvae added to each treatment

Chemical profile and fatty acid composition of extracts from *Mycale* spp.

By visual comparison of chromatograms obtained from HPLC, both MeOH and CHCl$_3$ extracts of *M. adhaerens* from Hong Kong and *M. laxissima* from the Bahamas differed in terms of number, retention time, and relative intensity of peaks (Fig. 4).

At 1× TLC, the total intensity of peaks revealed by GC-MS in the crude CHCl$_3$ extract of *Mycale laxissima* was higher than that of *M. adhaerens* (Fig. 5). The 2 extracts shared 48 peaks in common, while 20 and 29 unique peaks were only observed in the extracts of *M. adhaerens* and *M. laxissima*, respectively (Fig. 5, Table 6). For example, peaks with retention times of 11.44, 16.34, 17.94, and 21.06 min and identified as 2-hydroxy-4-hydroxylaminopyrimidine, methyl palmitate, methyl octadecanoate, and 3-methoxy-5a-morpholin-4-yl-5a,6,7,8,9,9a-hexahydrodibenzofuran-2-ol, respectively, were found in the extracts of both sponges. On the other hand, peaks with retention times of 14.23 and 16.18 min and identified as 1-dodecanol, 3,7,11-trimethyl and 11-hexadecenoic acid, methyl ester, respectively, were only present in the extract of *M. adhaerens*. Similarly, peaks with retention times of 17.19, 17.48, and 18.25 min and identified as cyclopropanebutanoic acid, heptadecanoic acid, and octadecanoic acid, 2-hydroxy-1,3-propanediyl ester were only observed in the extract of *M. laxissima*.

Using GC-MS, 41 and 30 fatty acids were detected in the CHCl$_3$ extracts of *Mycale adhaerens* and *M. laxissima*, respectively (Table 7). The most dominant fatty acid for both extracts was identified as 5,9-hexacosadienoic acid, which constituted 35.68 and 25.26% of the total fatty acids in *M. adhaerens* and *M. laxissima*, respectively. Although GC-MS also revealed a number of fatty acids in common for both extracts, their fatty acid profiles differed in terms of the type and relative abundance of fatty acids detected. The dominant fatty acids in the extract of *M. adhaerens* were long, straight-chain, unsaturated fatty acids, including 5,8,11,14,17-eicosapentaenoic acid, 5,9-hexacosadienoic acid, 9,19-hexacosadienoic acid, and 19-hexacosenoic acid (together representing 51.89% of the total fatty acids), while those in the extract of *M. laxissima* were straight-chained, saturated fatty acids, including hexadecanoic acid and octadecanoic acid, and the long, straight-chained, unsaturated fatty acid 5,9-hexacosadienoic acid (together comprising 50.36% of the total) (Table 7). Some fatty acids were detected in 1 of the sponge extracts only. For example, 4,7,10,13,16,19-docosahexaenoic acid and 9,10-methylene-octadecanoic acid were only found in the extracts of *M. adhaerens* and *M. laxissima*, respectively. For both extracts, branched fatty acids constituted only <10% of the total.

DISCUSSION

Our study demonstrated that geographically separated congeneric sponges have different surface-associated bacterial communities and chemical and fatty acid profiles, and their extracts show different antifouling activities. In this study, we first compared the bacterial communities on the surfaces of 2 congeneric sponges, *Mycale adhaerens* and *M. laxissima*, from 2 geographically separated regions, Hong Kong and the Bahamas, respectively, using both culture-dependent and -independent techniques. Despite the generally high variability of the bacterial communities associated with *Mycale* within one particular species (Table 2 in Lee & Qian 2003; Fig. 1, Table 3 in the

Table 5. *Mycale* spp. Terminal restriction fragments (TRF) derived from bacterial communities on surfaces of hydrogels of ddH_2O, $CHCl_3$ solvent, and antibiotics (A), and MeOH or $CHCl_3$ extracts of *M. adhaerens* and *M. laxissima*. Crude extracts were obtained by combining extracts from >1 sponge for each species. Hydrogels had been exposed to flow-through seawater in the natural environment for 72 h. Data are number of occurrences of a TRF in 4 replicates of a given sample. No data: total absence of TRF in a sample

Length of TRF (bp)	ddH_2O	$CHCl_3$	A	*M. adhaerens*		*M. laxissima*	
				MeOH	$CHCl_3$	MeOH	$CHCl_3$
38	2	2				4	
39	4	2	3	2	3	2	3
42				3	3	2	2
44						3	
45						4	
64						3	
68	2	3				2	3
80				3			
81				3	2	4	
90						4	
91	3	3					
92, 120						2	
123			2	3		3	2
125				3		2	
127				2	2	4	
128	3	3		3	3	4	
129	2	3					
149	2			2		2	
150						2	
151				2			
152, 176						2	
177	3	2	3			3	
182	2	2					
207						2	
208						3	
281	2						
299					2		
301				4	3		2
302							2
310, 311				4	3		
312						4	
313	2	2	2	3			
314	2	3					
315	4	4	3		2	3	4
367	2	3		3		2	
368	2	2		2	2		
370	2	3		3	2		
371				2		3	
373				3	3		
375	4	4	4	4	4	3	4
376	2	3				2	
379				2			
420					3		
421					3		
422	4	4		4		3	
423	3	2				3	
426					2		
Average (±SD) no. of TRFs	13.0 ± 2.4	12.5 ± 1.3	4.3 ± 1.3	16.0 ± 1.2	11.3 ± 2.6	21.0 ± 1.8	5.5 ± 1.3

present study), the sponge-associated bacterial communities were distantly related to the bacterial communities associated with the reference surfaces at their corresponding locations. Since the sponge surfaces are not 'uniform' and may consist of different micro-habitats with slightly different physical or chemical characteristics, the intraspecific variations may be attributed to the texture and topography of the sponge surfaces (Dexter et al. 1975), as well as the production of bioactive metabolites from the sponges and/ or sponge-associated microorganisms (Engel & Pawlik 2000). Sponge metabolites may recruit specific bacteria as indicated by the unique TRFs (Table 3), which benefit the sponges indirectly, or may defend the sponges against the colonization of harmful bacteria directly. The latter was supported by the antibacterial growth (Table 4) and antibacterial attachment activities (Figs. 2 & 3, Table 5) of the sponge extracts.

Using the traditional isolation procedure, more bacteria were isolated from the Hong Kong sponge than from the Bahamas sponge, and identification of these isolates by 16S rRNA gene sequencing revealed no bacterium in common on either surface (Table 1). It should be kept in mind that only a small proportion (<1%) of bacteria living in natural habitats is culturable using existing techniques (Hentschel et al. 2003). A comparison of bacterial communities based on the cultivation method can only reveal the culturable part of a community under the same selection pressure (i.e. culture conditions). Therefore, a culture-independent technique, TRFLP analysis, which can reflect a whole bacterial population, was also employed for the comparison. Using TRFLP analysis, the bacterial community on the Hong Kong sponge derived more ribotypes (Table 3) and was found to differ substantially from that on the Bahamas sponge (<20% similarity; Fig. 1). This observation contradicted the findings from previous studies suggesting that bacterial communities in sponges from different seas were similar (Hentschel et al. 2002, 2003), but concurred with our comparison based on the cultivation method in this study and with the findings from our laboratory on other congeneric sponge species, including *Callyspongia* (Qian et al. 2006) and *Halichondria* spp. (authors' unpubl. data). Taylor et al. (2005) also demonstrated that

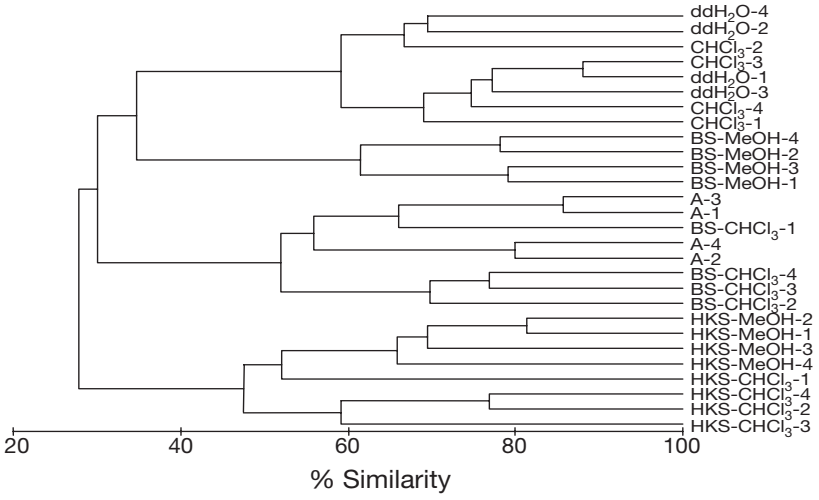

Fig. 3. *Mycale* spp. Dendrogram showing the similarity of bacterial communities on the surfaces of hydrogels of ddH_2O, $CHCl_3$ solvent, antibiotics (A), and MeOH or $CHCl_3$ extract of *M. adhaerens* (HKS) and of *M. laxissima* (BS). Hydrogels had been exposed to flow-through seawater in the natural environment for 72 h

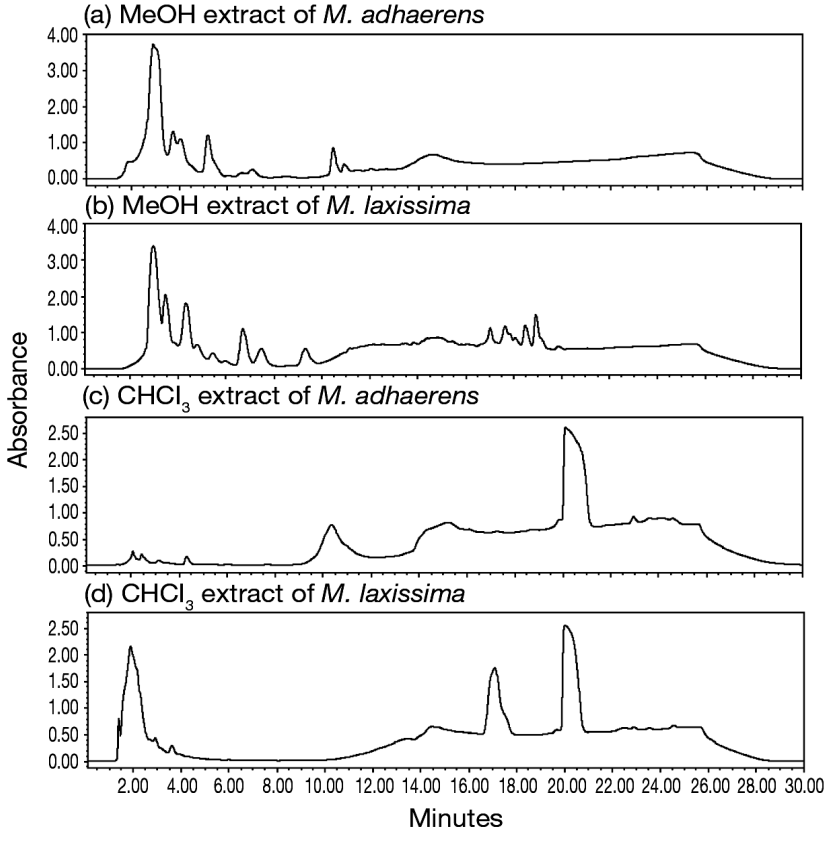

Fig. 4. *Mycale* spp. Chromatograms of HPLC of crude (a,b) MeOH and (c,d) $CHCl_3$ extracts of (a,c) *M. adhaerens* and (b,d) *M. laxissima*. HPLC was performed using a gradient from 5% aqueous CH_3CN to 85% CH_3CN at a flow rate of 1 ml min^{-1}; 10 µl of each extract at 20× tissue-level concentration were injected for analysis

the bacterial communities associated with the sponge *Cymbastela concentrica* from temperate and tropical regions were different, while bacterial communities in close proximity were more similar. These discrepancies are likely due to the methods used, which target different taxonomic levels. Using TRFLP analysis, a lower taxonomic level, down to ribotypes, is revealed, while, in other studies, the comparisons are at family, class, or even phylum levels. Bacterial communities on congeneric sponges that are geographically distant may show more differences than those on sponges in close vicinity. This could be due to the simple fact that the environmental factors (e.g. temperature, nutrient content, water flow, etc.) in geographically distant areas are often different, leading to different indigenous bacterial communities in the water column that eventually colonize sponges. Although bacterioplankton differ among different oceans (Hewson et al. 2006), the effects — in respective locations — of bacterial community differences in the water column on sponge–surface bacterial communities remain unknown. Further studies using clone libraries may help identify the types of bacteria that are specifically associated with different congeneric sponges.

Sponges show heterogeneity in the distribution of different types of associated microbes and metabolites among their tissues. For instance, photosynthetically active microbes such as cyanobacteria and eukaryotic algae are generally found in the outer layer or light-exposed part of sponges, while heterotrophic and autotrophic microbes populate the inner core or the mesophyl matrix of sponges (Hentschel et al. 2003). Kubanek et al. (2002) also demonstrated that different layers of sponges contained different types and amounts of bioactive metabolites. It would be interesting to study the degree of variability in chemical profiles and antifouling activities within intraspecific sponges. However, in our study, with the aim of comparing the chemical profiles and antifouling activities of congeneric species, several

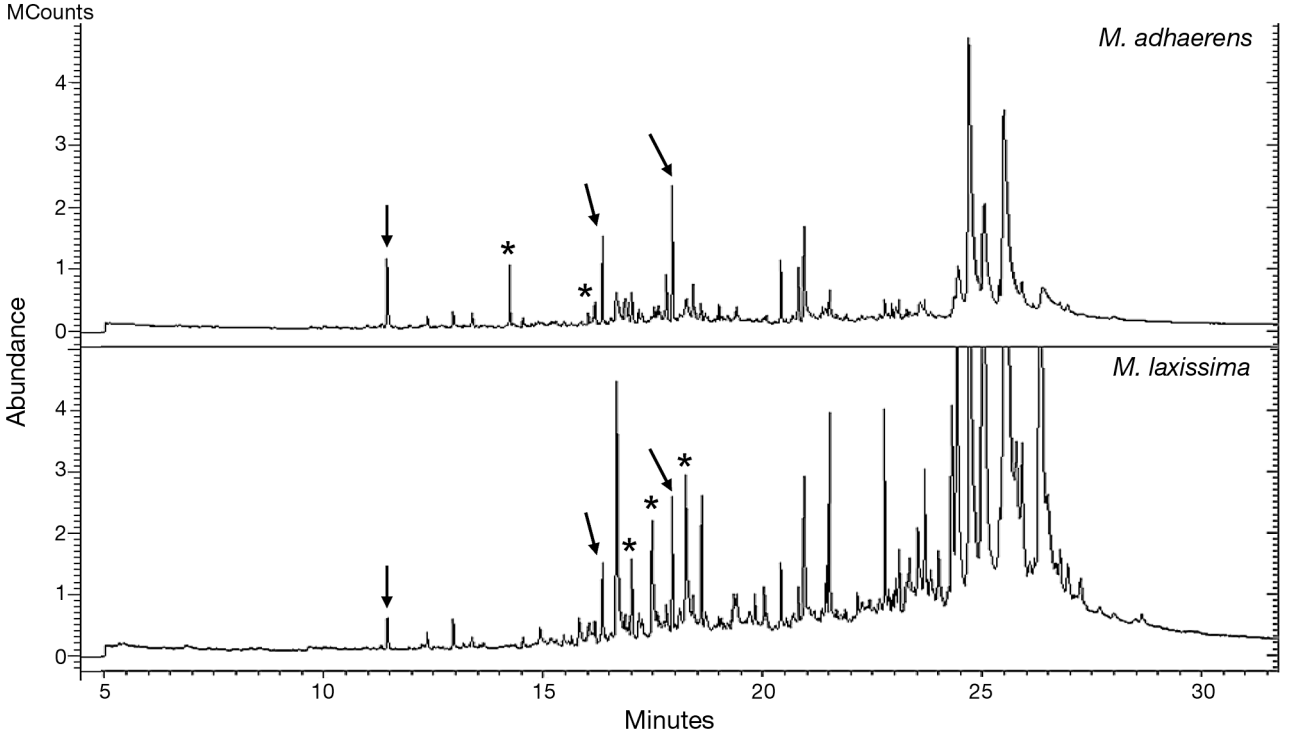

Fig. 5. *Mycale* spp. Chromatograms of GC-MS of crude $CHCl_3$ extracts of *M. adhaerens* (upper panel) and *M. laxissima* (lower panel). Extracts injected were at 1× tissue-level concentration (arrows: example of common peaks; *: examples of unique peaks present in particular extracts)

Table 6. *Mycale* spp. Unique peaks revealed by GC-MS in the $CHCl_3$ extracts of *M. adhaerens* from Hong Kong (HKS) and *M. laxissima* from the Bahamas (BS). Crude extracts were obtained by combining extracts from >1 sponge for each species. ✓: presence of a peak in a sample; no data: absence of a peak. Peaks were identified by the NIST GC-MS library and only matches with probability >25% are presented

Retention time (min)	Extract HKS	Extract BS	Closest match to the library	Probability (%)
10.99	✓		Benzaldehyde, 2,4,5-trimethyl-	36
18.27	✓		2-(3-Acetoxy-4,4,10,13,14,15,16,17-tetradecahydro)-1H-cyclopenta[a]phenanthren-17	35
18.74	✓		Hexadecanoic acid, 2-pentadecyl-1,3-dioxan-5-yl ester cis	28
20.68	✓		2-Methoxy-5a-morpholin-4-yl-5a,6,7,8,9,9a-hexahydrodibenzoguran-2-ol	48
23.23	✓		Cholesteryl benzoate	29
23.28	✓		9-Desoxo-9-x-acetoxy-3,8,12-tri-O-acetylingol	39
23.87	✓		2-(3-Acetoxy,4,10,13,14-pentamethyl)-2,3,4,5,6,7,10,11,12,13, 14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17	39
27.27	✓		1′,1′-Dicarboethoxy-1.beta.,2.beta.-dihydro-3H-cycloprop[1,2]cholesta-1,4,6-trien-3-one	33
15.82		✓	Methyl 12-methyl-tridecanoate	39
17.19		✓	Cyclopropanebutanoic acid	53
18.25		✓	Octadecanoic acid, 2-hydroxy-1,3-propanediyl ester	45
19.34		✓	Docosahexaenoic acid, 1,2,3-propanetriyl ester	48
19.60		✓	Milbemycin B, 5-demethoxy-5-one-6,28-anhydro-25-ethyl-13-chloro-oxime	44
19.83		✓	Hexanedioic acid, mono(2-ethylhexyl) ester	60
20.04		✓	Eicosanenitrile	34
20.71		✓	Indoxazen-5-ol-4-one, 3-[9-tridecenyl]	53
22.27		✓	7,8-Epoxylanostan-11-ol, 3-acetoxy-	47
22.52		✓	Cholest-7-en-6-one,3-(acetyloxy)-9-hydroxy-(3.beta.,5.beta.)	26
23.27		✓	8,14-Seco-3,19-epoxyandrostane-8,14-dione,17-acetoxy-3.beta.-methoxy-4,4-dimethyl	50
23.74		✓	Pagicerine	39

Table 7. *Mycale* spp. Fatty acid compositions of crude chloroform extracts of *M. adhaerens* from Hong Kong and *M. laxissima* from the Bahamas analyzed by gas chromatography–mass spectrometry (GC-MS). Crude extracts were obtained from combining extracts from >1 sponge for each species. i and a: iso-branched and anteiso-branched fatty acids, respectively. Data presented are percent fatty acids. Fatty acids representing <0.5% in both extracts were excluded (numbers in parentheses: positions of unsaturated bonds)

Fatty acids	Chloroform extract of: *M. adhaerens*	*M. laxissima*
Straight chain		
Saturated		
Pentadecanoic acid, 15:0	0.19	2.41
Hexadecanoic acid, 16:0	4.04	10.88
Heptadecanoic acid, 17:0	0.30	1.07
Octadecanoic acid, 18:0	3.45	14.22
Nonadecanoic acid, 19:0	0.24	0.40
Eicosanoic acid, 20:0	1.54	0.34
Docosanoic acid, 22:0	0.93	–
Tetracosanoic acid, 24:0	1.37	0.44
Unsaturated		
7-Hexadecenoic acid, 16:1(7)	1.32	1.59
6,9,12,15-Hexadecatetraenoic acid, 16:4(6,9,12,15)	0.83	0.63
7-Octadecenoic acid, 18:1(7)	0.91	2.14
9-Octadecenoic acid, 18:1(9)	1.61	2.97
5,8,11,14-Eicosatetraenoic acid, 20:4(5,8,11,14)	1.99	2.67
5,8,11,14,17-Eicosapentaenoic acid, 20:5(5,8,11,14,17)	5.66	1.31
9-Eicosenoic acid, 20:1(9)	1.01	1.65
5,13-Docosadienoic acid, 22:2(5,13)	0.80	2.99
4,7,10,13,16-Docosapentaenoic acid, 22:5(4,7,10,13,16)	0.56	–
4,7,10,13,16,19-Docosahexaenoic acid, 22:6(4,7,10,13,16,19)	3.77	–
5,9-Tricosadienoic acid, 23:2(5,9)	0.71	1.82
9-Tetracosaenoic acid, 24:1(9)	3.21	0.59
15-Tetracosaenoic acid, 24:1(15)	0.81	1.12
5,9-Pentacosadienoic acid, 25:2(5,9)	3.10	1.44
19-Hexacosenoic acid, 26:1(19)	4.96	0.71
5,9-Hexacosadienoic acid, 26:2(5,9)	35.68	25.26
9,19-Hexacosadienoic acid, 26:2(9,19)	5.59	2.55
5,9,19-Hexacosatrienoic acid, 26:3(5,9,19)	0.73	–
Branched		
Saturated		
12-Methyl-tridecanoic acid, i14:0	0.80	1.93
13-Methyl-tetradecanoic acid, i15:0	0.16	0.72
14-Methyl-pentadecanoic acid, i16:0	0.28	0.30
14-Methyl-heptadecanoic acid, a17:0	0.46	0.31
15-Methyl-heptadecanoic acid, i17:0	0.40	0.20
3-Methoxy-heptadecanoic acid, 3-methoxy 17:0	0.55	–
16-Methyl-octadecanoic acid, a19:0	0.13	0.89
Unsaturated		
3-Methoxy-docosadienoic acid, 3-methoxy 20:2	4.22	0.58
Cyclic		
9,10-Methylene-octadecanoic acid, 19:0 cyclo w9c	–	2.26

colonies from each species were combined, extracted, and analyzed in order to minimize bias introduced based on the comparisons of 1 sponge colony from each species.

The crude extracts from the 2 sponges were tested for antibacterial activity against bacterial isolates from the reference and sponge surfaces. Disc diffusion assays revealed that the $CHCl_3$ extract from the Hong Kong sponge inhibited the growth of half of the bacteria isolated from the reference surface from Hong Kong, but not any of those isolated from the Bahamas reference surface (Table 4). Similarly, Newbold et al. (1999) demonstrated that different sponges from the Caribbean produced different antibacterial compounds that targeted different bacterial strains. Kelman et al. (2001) also reported that extracts from sponges only affected the growth of indigenous bacteria isolated from the water column, but not the growth of sponge-associated bacteria. Besides, the extracts were embedded in phytagels at TLC and tested for anti-bacterial attachment activity. It should be noted that this concentration may not exactly be the concentration that naturally occurred in the sponge tissues, since the distribution of compounds in the sponges was unknown in the present study. However, assuming that compounds are distributed evenly throughout the tissue volume, it is likely an ecologically meaningful concentration, and is a widely accepted measurement for ecological studies (Jensen et al. 1996). In the phytagel assays, certain bacterial types, as indicated by specific TRFs, were excluded from the surfaces of hydrogels containing extracts of different sponges (Table 5). All of the sponge extracts altered the bacterial communities on hydrogel surfaces to certain extents (Fig. 3). These results indicate that the 2 congeneric *Mycale* spp. possess anti-microfouling activity, which functions by inhibiting bacterial growth or attachment. The activity is specific to ecologically relevant bacteria, which may explain the differences in bacterial communities associated with the surfaces of the 2 sponges.

Larvae of an important fouling polychaete *Hydroides elegans* in Hong Kong waters responded differently to the hydrogels containing extracts of different sponges (Fig. 2b). This polychaete exists in the same habitat as

Mycale adhaerens, but fouling by this species has not been observed on the sponge surface. An indirect chemical defense mechanism against larval settlement, via modulating the surface bacterial community of the sponge, was thus proposed. To test this hypothesis, the filmed hydrogels were subsequently exposed to the larvae. Hydrogels with bacterial films altered by the $CHCl_3$ and MeOH extracts from Hong Kong sponge, not only killed the larvae, but also inhibited larval settlement, while those with bacterial films altered by extracts from the Bahamas sponge did not. The anti-larval settlement effect of the Hong Kong sponge extracts may be attributed to the extracts themselves or to the bacterial films altered by these extracts. However, the former is unlikely because none of the larvae were killed, nor was larval settlement inhibited when the bacterial films on the hydrogels were removed (data not shown). In fact, larvae of *H. elegans* respond differently to different mono-species bacterial films (Lee & Qian 2003) and were sensitive to the bacterial species composition and the bacterial density of the films (Huang & Hadfield 2003). Therefore, the extracts from the Hong Kong sponge likely alter the bacterial communities by changing bacterial species composition and/or bacterial density on hydrogel surfaces, which, in turn, controls larval settlement of *H. elegans*; an indirect defense mechanism against fouling by *H. elegans* is thus proposed. Again, these results support our assertion that the bioactivity of sponge extracts is species specific. It would be equally interesting if a major fouling organism in the Bahamas could also be included in the bioassays, which could provide a more comprehensive assessment of the antifouling activity of extracts against ecologically relevant organisms.

In order to correlate the observed differences in surface bacterial communities and antifouling activities to the chemical compositions of the 2 congeneric sponges, the sponge tissue extracts were subjected to HPLC and GC-MS analyses. Our results indicate that the chemical compositions of the crude extracts from the 2 sponges differ, evidenced by different peak patterns in HPLC and GC-MS chromatograms (Figs. 4 & 5, Table 6). In addition, fatty acid profiles for the extracts of the 2 *Mycale* sponges differed and the Hong Kong sponge had a more diverse fatty acid composition (Table 7). The dominant fatty acid for both sponges was 5,9-hexacosadienoic acid, which is a major fatty acid in Demospongia (Ando et al. 1998). Surprisingly, there has been no report on bioactivity of this fatty acid. Other monosaturated fatty acids, including 7-hexadecenoic acid, 7-octadecenoic acid, and 9-octadeceonic acid, were found in both sponges. As these fatty acids are typical fatty acids of cyanobacteria, fungi, and microalgae (Harwood & Russell 1984), they are possibly of microbial origin in both sponges. Some fatty acids, for instance, the polyunsaturated fatty acids 5,8,11,14,17-eicosapentaenoic acid, 4,7,10,13,16,19-docosahexaenoic acid, and 4,7,10,13,16-docosapentaenoic acid and the branched fatty acids 3-methoxy-heptadecanoic acid, 17-methyl-octadecanoic acid, 13-methyl-eicosanoic, and 3-methoxy-docosadienoic acid, were found or were highly abundant only in the Hong Kong sponge (Table 7). The long-chained polysaturated fatty acids and the branched fatty acids are characteristic of microalgae and bacteria, respectively (Harwood & Russell 1984), suggesting possible involvement of associated microbes of the Hong Kong sponge in the production of these fatty acids. The polyunsaturated fatty acids 5,8,11,14,17-eicosapentaenoic acid and 4,7,10,13,16,19-docosahexaenoic acid were reported to be toxic to an anostracan grazer (Jüttner 2001) and to ameliorate murine acute renal failure (Kielar et al. 2003), respectively, but unfortunately there has been no reference in the literature to the other specific polysaturated and branched fatty acids. On the other hand, the Bahamas sponge had a higher proportion of saturated fatty acids (35.7 vs. 16.7%), dominated by hexadecanoic acid and octadecanoic acid, but a much lower proportion of unsaturated fatty acids (48.4 vs. 77.2%) than the Hong Kong sponge (Table 7). In addition, a cyclic fatty acid, 9,10-methylene-octadecanoic acid, was detected only in the Bahamas sponge. Again, we know relatively little about the bioactivity of these fatty acids. At present, there is only 1 study that has reported on the fatty acids isolated from a *Mycale* sp. (Carballeira et al. 1992), but bioactivity of the fatty acids was not measured.

The present study has demonstrated that the bacterial communities on the surfaces of 2 congeneric *Mycale* sponges, one from Hong Kong and the other from the Bahamas, differed substantially, and the 2 sponges showed different bioactivities in inhibiting bacterial colonization and the larval settlement of *Hydroides elegans*. These differences may simply be due to the geographical separation of the 2 sponges. However, the differences in the production of bioactive metabolites, particularly the fatty acid composition, as shown by different chemical compositions of the crude extracts of the 2 sponges, may also contribute to the differences in the surface bacterial communities. In reverse, the differences in the surface bacterial communities may result in the differences in chemical compositions, since different associated bacteria would produce different metabolites or fatty acids with different functions, resulting in different sponge bioactivities. Our results also suggest the possible involvement of associated microbes in the production of fatty acids in sponges; therefore, isolation of associated microbes

and analysis of their fatty acid profiles might help verify this hypothesis. Furthermore, future investigations involving the isolation and testing of purified active compounds and fatty acids from these sponges may help us understand the complex interactions between bioactive compound production, surface bacterial community structure, and the antifouling activity of sponges.

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