

# Consistent Bacterial Community Structure Associated with the Surface of the Sponge *Mycale adhaerens* Bowerbank

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## Abstract

As a crucial step in the identification of possible association between bacteria and sponges, we investigated if a unique bacterial population community was consistently associated with the surface of the sponge *Mycale adhaerens*, irrespective of environmental conditions. The composition of bacterial communities associated with the surface of sponges at three geographically distinctive sites in Hong Kong waters over four seasons was examined by analyzing terminal restriction fragment length polymorphism of the bacterial 16S rRNA genes. Statistical analysis indicated that bacterial communities on inanimate reference surfaces (polystyrene dishes deployed in the close vicinity of the sponge colonies for 7 days) had a relatively high degree of both site and seasonal specificities ( $R$  statistics of pairwise comparisons  $\sim 1$ ), which might be attributed to the differences in environmental conditions at different sites and seasons. On the contrary, the sponge-surface-associated bacterial communities from different sites and seasons were hardly distinguishable from each other (lowest  $R = -0.16$ ) but were rather distinctive from the reference bacterial communities ( $R \sim 1$ ), suggesting a highly stable and distinctive bacteria-sponge association irrespective of the environmental conditions. The occurrence of some unique bacterial types in the sponge-surface-associated communities over space and time suggests that the associations are consistent and specific.

## Introduction

Marine sponges (Phylum: Porifera) are sessile organisms that effectively filter out microorganisms and small

particles from the surrounding water. Whereas most “captured” microorganisms serve as food particles and are digested by phagocytotically active cells in sponge, the others are retained, even thrive to reach a balanced state, and finally form specific associations with sponges. Therefore, sponge-microorganism associations become a common phenomenon in nature. For instance, unicellular algae [84], cyanobacteria [82], dinoflagellates [32], zooxanthellae [72], zoochlorellae [34, 95], and members of the domain Archaea [29, 65] have been reported as associates in sponges. Among these microorganisms, bacteria are the most dominant group that constituted up to 40% of the biomass or 60% of the tissue volume of certain sponge species [70, 84, 89, 90].

Sponge-bacterium associations promote mutual proliferation of nutrients and digestion and recycling of materials [7, 91]. Furthermore, bacteria in the sponges are the biosynthetic origins of highly diverse secondary metabolites that are potent antibacterial [49], antifeeding [96], and allelopathic [23] compounds. These metabolites provide chemical defense against predation, microbial attachment, and fouling by other invertebrates for the sponges [52]. In addition, sponges serve as a shelter for bacteria against grazers or the substrate for bacteria to attach so that bacteria can proliferate more rapidly because sponge metabolites can serve as a consistent nutrient supply [43]. There has also been evidence supporting the hypothesis that bacteria associated with sponge surfaces help sponge defend against colonization by other harmful organisms [36, 41, 44, 49, 53, 54, 79].

Earlier studies of sponge-bacterium associations mainly relied on morphological descriptions using transmission electron microscopy [84] and numerical taxonomy using cultivation [71]. However, these methods generally suffer from the fact that bacteria are largely indistinguishable by morphological characteristics, and

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that <1% of bacteria are cultivable from marine sponges [39]. Recent advances in molecular techniques have provided culture-independent tools to reveal the diversity and precise phylogenetic affiliation of bacteria associated with sponges by targeting the 16S rRNA genes in the bacterial community under investigation. A series of remarkable investigations by Friedrich *et al.*, using fluorescence *in situ* hybridization, denaturing gradient gel electrophoresis (DGGE), and the construction of DNA libraries, have shown that: (1) bacterial community in the Mediterranean sponge *Aplysina cavernicola* is dominated by bacteria with low guanine-cytosine content, including the delta- and gamma-Proteobacteria and representatives of the *Bacteroides* cluster [27]; (2) bacterial community in a congeneric sponge *Aplysina aerophoba* was surprisingly resistant to environmental perturbations [28, 83]; and (3) some bacteria in the phylogenetically distantly related sponges (*A. aerophoba* and *Thenoella swinhoei*) in nonoverlapping geographical locations are highly similar [38].

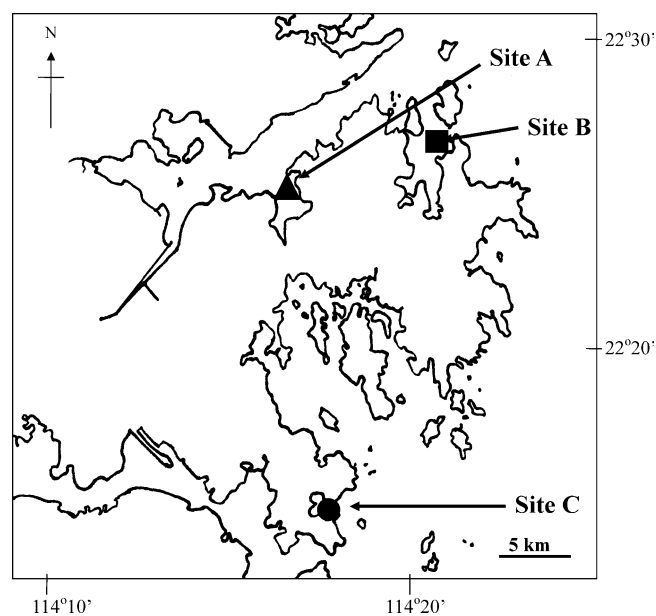
In contrast to the extensive studies on the bacterial community in the mesohyl of sponges for years [28, 38, 83, 84, 90], the description of the bacterial community associated with sponge surfaces is scarce. Moreover, past investigations generally employed a snapshot sampling strategy [57, 71, 92] that often does not allow an insight into the composition of sponge-associated bacterial communities over time. The present study investigated the compositional stability of the bacterial communities associated with the surface of the sponge *Mycale adhaerens* commonly found in Hong Kong waters. We compared the variations of bacterial community compositions on both the sponge and reference surfaces (i.e., polystyrene dishes deployed in the close vicinity of the sponge colonies for 7 days) in different seasons and at different sites by analyzing terminal restriction fragment length polymorphism (TRFLP) of 16S rRNA genes in bacterial community DNA. This method has been used successfully for the characterization of bacterial communities in marine samples [35, 60, 66] and has certain advantages over DGGE and 16S rRNA gene cloning in terms of its effectiveness, sensitivity, and consistency in differentiating microbial communities [9, 30, 60]. The variable and stable fractions of the bacteria communities were distinguished, and the possible identities of the stable bacterial associates were inferred using reference terminal restriction fragments (TRFs) derived from bacteria isolated from ecological relevant sites.

## Materials and Methods

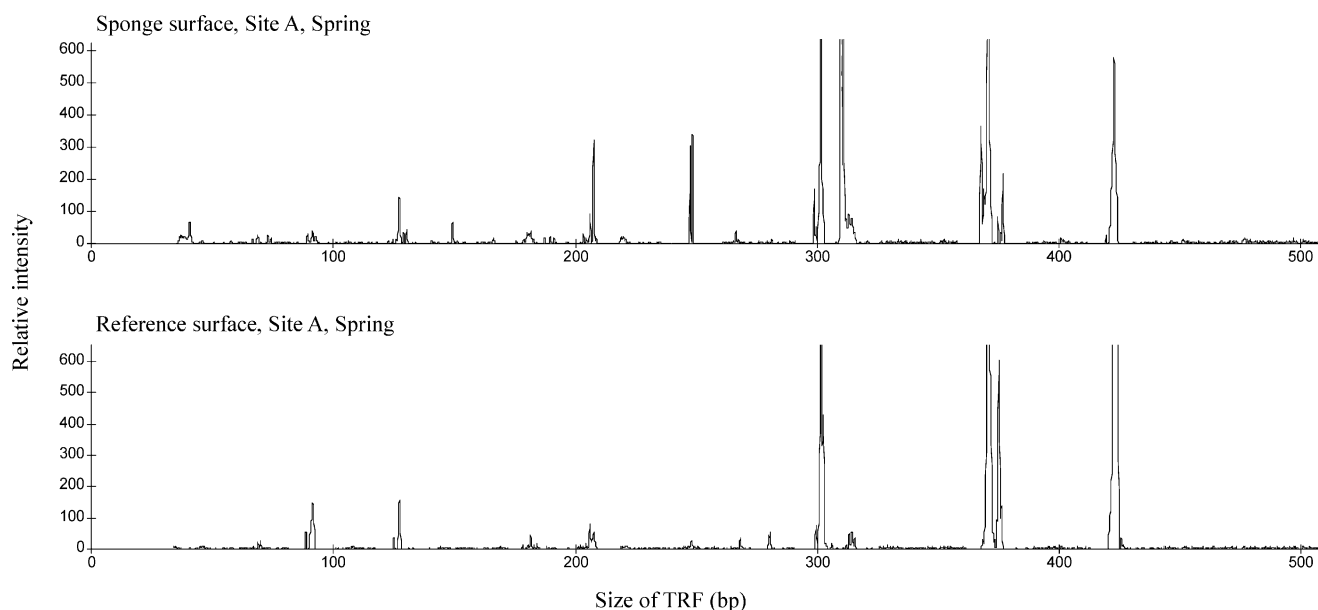
**Sampling Site and Time.** Bacterial communities were sampled from the surfaces of the sponge *M. adhaerens* and polystyrene petri dishes (as inanimate reference) at three sites in the eastern Hong Kong waters, namely, Three

Fathoms Cove (site A, Fig. 1), Long Harbour (site B, Fig. 1), and Clear Water Bay (site C, Fig. 1), in April (spring), July (summer), October 2003 (autumn), and January 2004 (winter). The three sites are at least 11.7 km away from each other, and environmental variables are monthly monitored by the Environmental Protection Department of Hong Kong SAR government year around. Site A continuously receives discharges from the numerous water catchments in the area and has the lowest salinity (30.9 ppt), but has the highest temperature (24.7°C), nutrient contents (total nitrogen: 0.19 mg L<sup>-1</sup>, phosphorus: 0.03 mg L<sup>-1</sup>), pH (8.3), and chlorophyll *a* content (3.55 µg L<sup>-1</sup>) among the three sites. Site B is a relatively open area receiving prevailing easterly winds throughout the year with the lowest temperature (24.0°C), pH (8.1), dissolved oxygen (6.3 mg L<sup>-1</sup>), and total nitrogen (0.13 mg L<sup>-1</sup>) and phosphorus content (< 0.02 mg L<sup>-1</sup>). Site C is a small enclosed bay area (opening of the bay is about 280 m) with relatively stagnant water, which has the highest salinity (32.3 ppt) but the lowest chlorophyll *a* content (1.66 µg L<sup>-1</sup>).

**Collection and Extraction of Bacterial Community DNA.** During each sampling, four colonies of *M. adhaerens*, each with a surface area of ca. 40 cm<sup>2</sup>, were rinsed with autoclaved 0.22-µm-filtered seawater. The surfaces were swabbed with sterile cotton tips to collect bacterial community. Each cotton tip was immediately frozen in 0.8 mL of extraction buffer (100 mM of Tris-HCl, 100 mM of Na<sub>2</sub>-EDTA, 100 mM of Na<sub>2</sub>HPO<sub>4</sub>, 1.5 M of NaCl, 1% of cetyl trimethylammonium bromide; at



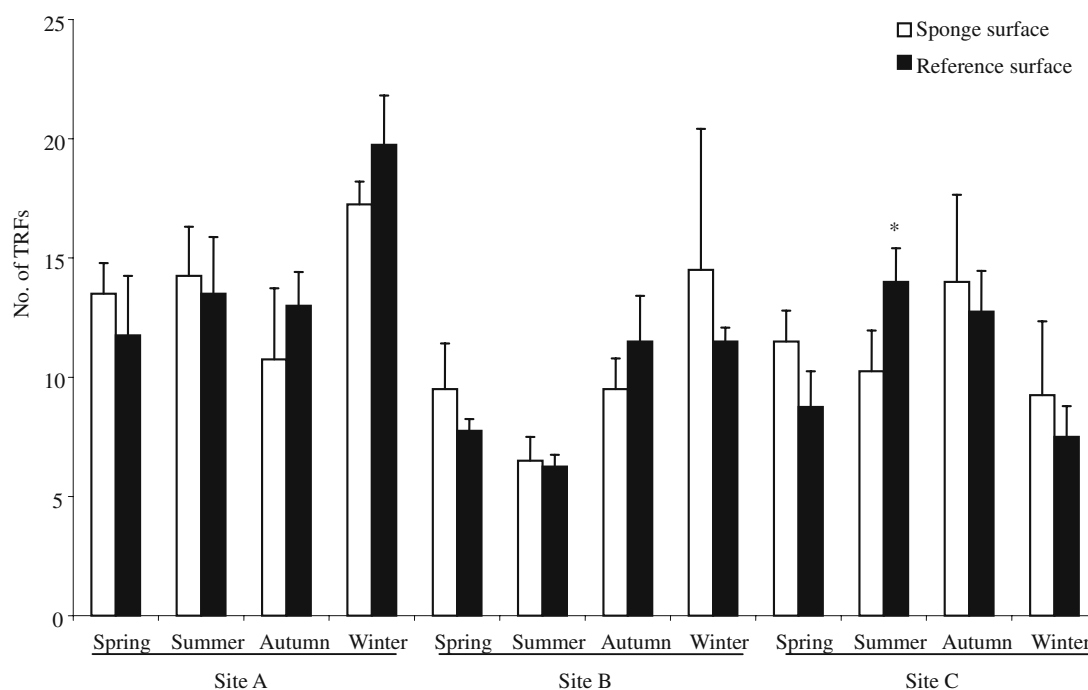
**Figure 1.** The location of sampling sites in the eastern Hong Kong waters. Site A: Three Fathoms Cove (22°26.91' N, 114°16.06' E); site B: Long Harbour (22°27.33' N, 114°21.00' E); site C: Clear Water Bay (22°15.92' N, 114°17.41' E).



**Figure 2.** Representative electropherograms of TRFs derived from *MspI* digestion of PCR-amplified bacterial community DNA obtained from the sponge (upper panel) and reference surfaces (lower panel). Samples for the sponge and reference surfaces were collected from site A during the first sampling in spring.

pH 8) and transported to the laboratory. Reference bacterial communities were collected analogously from presterile polystyrene dishes, which had been deployed in the close vicinity (2 m) of the sponge colonies 7 days prior to the sampling. Bacterial cells in the samples were lysed in

two freeze–thaw cycles using liquid nitrogen and a 65°C water bath. Total DNA was extracted and purified according to the sodium-dodecyl-sulfate-based method described in [99]. Purified DNA was dissolved in 50  $\mu\text{L}$  of sterile  $\text{H}_2\text{O}$  and kept at  $-20^\circ\text{C}$  until use.



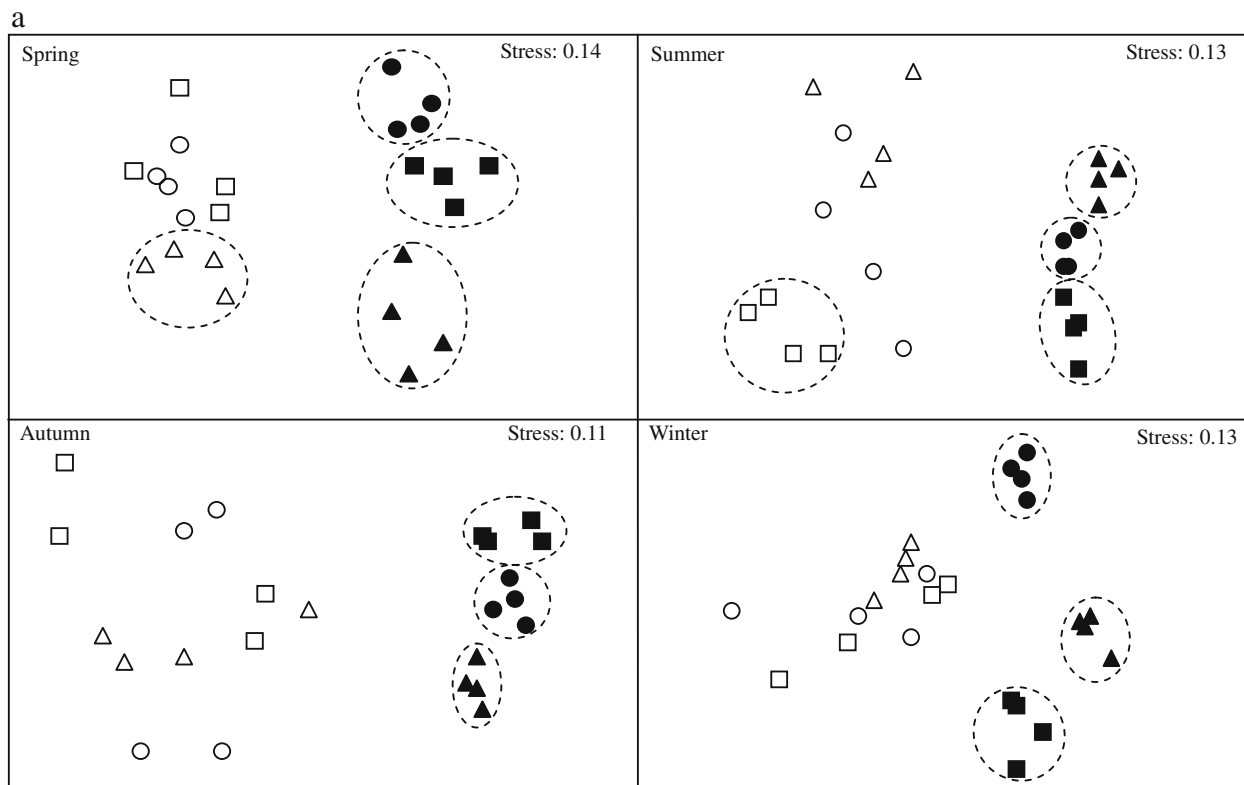
**Figure 3.** Number of TRFs derived from *MspI* digestion of PCR-amplified bacterial community DNA obtained from the sponge (open bars) and reference surfaces (closed bars) from different locations at different times. Data are expressed as the mean + 1 SD of four replicates. \*Data that are significantly different from the sponge surface in *t*-test.

**Polymerase Chain Reaction.** The 16S rRNA genes in the DNA samples were amplified by polymerase chain reaction (PCR) using the primers 341F (5'-CCTACG GGAGGCAGCAG-3') and 926R-Fam (5'-CCGTCAATT CCTTTRAGTTT-3') [55]. The latter was labeled at the 5' end with 6-carboxy fluorescein (Fam). Each PCR mixture contained 5  $\mu$ L of DNA sample, 1.25 U of *Taq* polymerase (Amersham Biosciences, USA), 0.25 mM of dNTPs, 0.1  $\mu$ M of each primer, 1.5 mM of MgCl<sub>2</sub>, and 2.5  $\mu$ L of 10  $\times$  PCR buffer in a total volume of 25  $\mu$ L. PCR was performed in the following thermal cycles: 95°C for 2 min (initial denaturation); 10 touchdown cycles of 95°C for 1 min (denaturation), 66°C (reduced to 57°C in increments of 1°C/cycle) for 1 min (annealing), and 72°C for 1 min (extension); additional 20 cycles with constant annealing temperature of 56°C; and 72°C for 5 min (final extension).

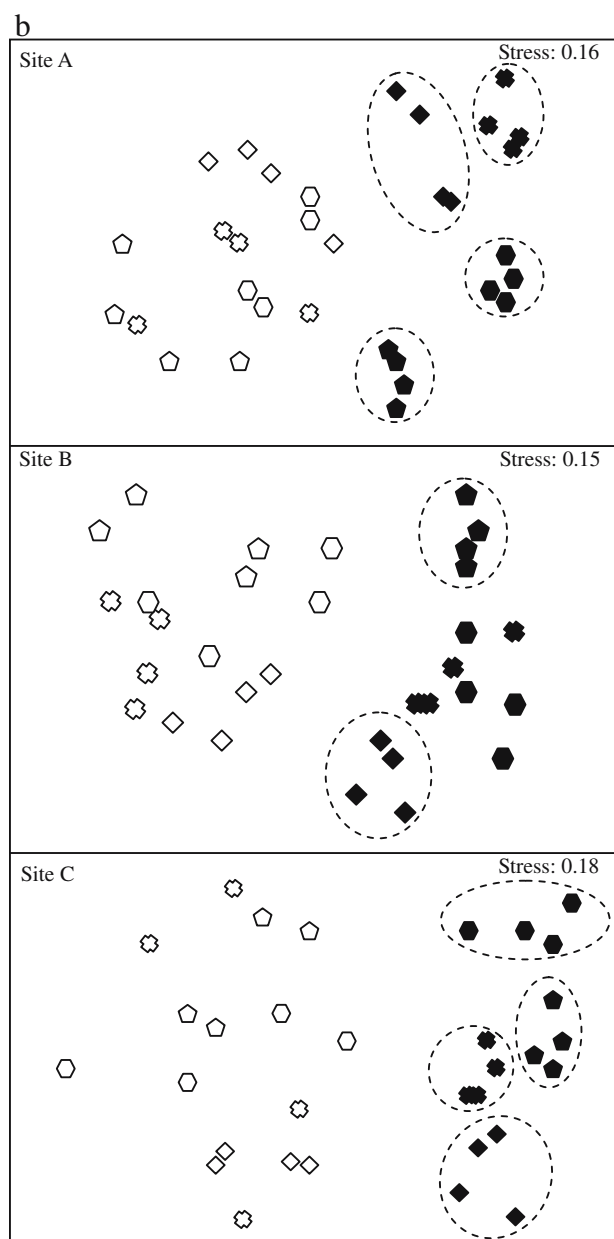
**TRFLP Analysis of Bacterial Communities.** Polymerase chain reaction products were cleaved with 10 U of the restriction enzyme *Msp*I at 37°C for 6 h. Cleaved PCR products were kept at -20°C until purification using the Wizard<sup>®</sup> PCR preps DNA purification system (Promega, USA). Ten microliters of purified products together with 0.5- $\mu$ L internal size standard (ET550-R,

Amersham Biosciences) were denatured at 95°C for 2 min, snap cooled on ice, and subjected to electrophoresis on a MegaBACE genetic analyzer (Amersham) operated in the genotyping mode. After electrophoresis, the size of the fluorescently labeled TRFs was determined by comparison with the size standards (ET550-R, Amersham Biosciences) using the software Genetic Profiler (Amersham Biosciences). TRFs that were < 50, 100, or 250 fluorescence units in intensity, < 35 bp in size, and > 550 bp in size were excluded from statistical analysis to screen off background noise, to avoid pseudo-TRFs derived from primers, and to avoid inaccurate size determination, respectively [35, 60].

**Estimation of Phylogenetic Affiliation of Bacteria in the Samples.** To estimate the phylogenetic affiliation of the bacteria from which the TRFs were derived, the size of TRFs derived from the samples was compared to those derived from 20 bacterial strains that had been isolated from the surface of *M. adhaerens* [53]. Extraction of crude DNA from each individual bacterial strain followed [85], while the primers, restriction enzymes, and PCR conditions to obtain TRFs from each isolate were the same as mentioned above. In addition, an on-line analysis from the Ribosomal Database Project (RDP;



**Figure 4.** MDS ordinations of bacterial communities (4 replicates each) associated with the sponge surface (open symbols) and the reference surface (filled symbols) from (a) different sampling sites (Site A: triangles; Site B: squares; Site C: circles) at a particular sampling time, or from (b) different sampling times (Spring: rhombuses; Summer: crosses; Autumn: pentagons; Winter: hexagons) at a particular sampling site. The circles in dotted-line indicate sample groups of relatively high similarity.



**Figure 4.** Continued.

<http://wcdm.nig.ac.jp/RDP/html/analyses.html>) [14] was employed to estimate the possible identity of the bacteria from which TRFs of interest were derived.

**Statistical Analysis.** The number of TRFs derived from the sponge and reference surfaces was compared by *t*-test. The similarity of TRF profiles among different samples was determined using nonmetric multidimensional scaling (MDS) and one-way analysis of similarities (ANOSIM) based on the size of the TRFs. Because PCR amplification methods may be biased toward numerically dominant bacteria or result in differential amplification that varies among taxa [81], the relative

fluorescence intensities of TRFs may not be accurate measures of the relative abundances of population members within a given sample and were not used in the statistical analysis. Instead, similarity matrices based on the total number of TRFs observed in all samples and the presence or absence of these TRFs in individual samples were generated. The rank orders of similarity between different samples given in the similarity matrix were used to calculate a two-dimensional ordination. The degree of disagreement between the rank-order distances among bacterial communities as displayed by the MDS plot and the real distances as derived from the similarity matrix is defined by the stress value [13]. Furthermore, global differences among individual samples were tested by one-way ANOSIM that is a nonparametric analog to analysis of variance (ANOVA) without the assumption of normality and homogeneity [11]. Pairwise comparisons tested for differences between samples when ANOSIM was significant. The ANOSIM statistic *R* is calculated based on the difference of mean ranks between groups (*r*<sub>B</sub>) and within groups (*r*<sub>W</sub>) and lies between -1 and +1:

$$R = (r_B - r_W) / (N(N - 1) / 4)$$

*R* values > 0.75 are generally interpreted as well separated, *R* > 0.5 as overlapping but clearly different, and *R* < 0.25 as barely separable at all [12]. Calculations were performed with the PRIMER v3.1 computer program (Plymouth Marine Laboratory, UK).

## Results

**TRF Profiles of Bacterial Communities.** Figure 2 showed the representative electropherograms of TRFs derived from *Msp*I digestion of PCR-amplified bacterial community DNA obtained from the sponge and reference surfaces. Under the combination of primers and restriction enzyme used in this study, the mean number of discernible TRFs derived from each type of samples ranged from 6 to 20 (Fig. 3). Generally speaking, numbers of TRFs derived from samples collected from site A (a total of 455 TRFs) and in winter (a total of 319 TRFs) were the highest among sites and seasons. For most of the cases, number of TRFs derived from the sponge and reference surfaces did not differ from each other significantly (Fig. 3).

For temporal variation of bacterial community composition, MDS ordinations arranged the bacterial communities obtained from each season into two distinctive groups: one for the sponge surface and one for the reference surface (stress value = 0.11–0.14; Fig. 4a). The bacterial communities derived from the reference surface were further divided into three subgroups, each contain-

**Table 1.** Pairwise comparison of similarity (ANOSIM) among bacterial communities obtained from (a) three sampling sites (A: Three Fathoms Cove; B: Long Harbour; C: Clear Water Bay) at different sampling times and (b) four sampling times at different sampling sites

			(a)							
			Reference surface			Sponge surface				
			A	B	C	A	B	C		
Spring	Reference surface	A								
		B	0.86							
		C	0.94	0.68						
	Sponge surface	A	0.99	1.00	1.00					
		B	0.93	0.98	1.00	0.58				
		C	1.00	0.99	1.00	0.92	0.42			
Summer	Reference surface	A								
		B	1.00							
		C	0.98	0.80						
	Sponge surface	A	1.00	1.00	1.00					
		B	1.00	1.00	1.00	0.98				
		C	0.98	0.87	0.87	0.48	0.52			
Autumn	Reference surface	A								
		B	1.00							
		C	0.96	0.91						
	Sponge surface	A	0.96	0.98	0.97					
		B	0.84	0.92	0.90	0.18				
		C	1.00	1.00	1.00	0.60	0.35			
Winter	Reference surface	A								
		B	1.00							
		C	1.00	1.00						
	Sponge surface	A	1.00	1.00	1.00					
		B	0.86	0.85	0.80	0.32				
		C	0.87	0.85	0.89	0.35	0.08			
			(b)							
			Reference surface				Sponge surface			
			Spring	Summer	Autumn	Winter	Spring	Summer	Autumn	Winter
Site A	Reference surface	Spring								
		Summer	0.85							
		Autumn	0.99	1.00						
		Winter	0.96	1.00	1.00					
	Sponge surface	Spring	0.99	1.00	1.00	1.00				
		Summer	0.99	1.00	1.00	1.00	0.77			
		Autumn	1.00	1.00	0.96	1.00	0.98	0.85		
Site B	Reference surface	Spring								
		Summer	0.97							
		Autumn	1.00	0.98						
		Winter	0.99	0.77	1.00					
	Sponge surface	Spring	0.98	1.00	1.00	1.00				
		Summer	1.00	1.00	1.00	1.00	0.54			
		Autumn	1.00	1.00	0.92	0.99	0.85	0.35		
Site C	Reference surface	Spring								
		Summer	0.60							
		Autumn	1.00	0.73						
		Winter	1.00	1.00	0.99					
	Sponge surface	Spring	1.00	1.00	1.00	1.00				
		Summer	0.89	0.87	0.96	0.96	0.5			
		Autumn	0.99	1.00	1.00	1.00	0.98	0.70		
		Winter	0.90	0.82	0.85	0.92	0.76	0.28	0.59	

TRFs that were < 50 fluorescence units in intensity, < 35 bp in size, or > 550 bp in size were excluded from the analysis. Values indicated are *R* statistics of pairwise comparisons. When *R* value is close to 1, it indicates that intersample variation is larger than intrasample (i.e., replicates) variation, whereas *R* value close to 0 indicates that there is no difference between inter- and intrasample variations. Significant *R* values are bold-typed ( $P < 0.05$ ).



**Table 2. Summary of occurrence of common TRFs**

Sampling time	TRF observed on both sponge and reference surfaces in all sites (bp)							TRF observed exclusively on sponge surfaces in all sites (bp)							TRF observed exclusively on reference surfaces in all sites (bp)					
	301	302	370	371	375	422	423	150	247	248	249	310	311	419	420	300	301	303	370	422
Spring		✓		✓	✓		✓			✓	✓	✓	✓					✓	✓	
Summer		✓			✓				✓	✓		✓	✓				✓			
Autumn	✓	✓	✓		✓				✓	✓		✓	✓	✓		✓				
Winter		✓	✓		✓	✓		✓	✓	✓		✓	✓		✓					✓

‘✓’ indicates a particular TRF observed in all three sampling sites (A, B, and C) and with an intensity >50 fluorescence units.

ing all replicate samples of the same site. The bacterial communities from the sponge surface showed no clear distinction among the individual sites (Fig. 4a). Similar grouping was obtained when the bacterial communities derived from each site were analyzed for seasonal variations; the sponge bacterial communities were all clearly separated from the reference bacterial communities (stress value = 0.15–0.18; Fig. 4b). Whereas the reference bacterial communities could be further grouped into separate seasons, the sponge bacterial communities showed no clear distinction among the seasons (Fig. 4b). The results of MDS ordinations were further supported by those of ANOSIM that detected significant differences between the sponge and reference samples as well as among the reference samples ( $R$  values = 0.60–1.00; Table 1) and that showed no clear distinction among the sponge samples from different sites and seasons ( $R$  values = 0.08–0.98; Table 1). Similar observation was found when TRF threshold intensity was set at 100 or 250 fluorescence units in statistical analysis (data not shown),

and therefore, only the data set generated based on the threshold intensity at 50 fluorescence units was presented.

Table 2 summarizes the occurrence of common TRFs among samples. The TRFs at 302 and 375 bp were found at all sampling sites and at all sampling times in both the sponge and reference samples. Some TRFs (e.g., 301, 370, 371, 422, and 423 bp) were found on both the sponge and reference samples from all sites for at least one sampling time (Table 2). The TRFs at 248, 310, and 311 bp were observed exclusively on the sponge surface at all sampling sites and at all sampling times (Table 2). No such exclusive distinctive TRFs were observed for the reference samples. Some other TRFs were associated with either the sponge (e.g., 150, 247, 249, 419, and 420 bp) or the reference samples (e.g., 300, 301, 303, 370, and 422 bp) at least at one sampling time (Table 2).

**Phylogenetic Affiliation of Bacteria in the Samples.** In total, 13 out of 20 bacteria isolated from the surface of the sponge in our previous study [53]

**Table 3. TRFs derived from *MspI* digestion of PCR-amplified 16S rRNA genes obtained from individual bacterial strains isolated from the surface of *Mycale adhaerens***

Isolate	Accession no.	Closest match of isolate	Phylogenetic branch	Length of TRF derived from <i>MspI</i> digestion (bp)
S6	AY241401	<i>Pseudoalteromonas spongiae</i>	$\gamma$ -Proteobacteria	129
S4	AY241399	<i>Bacillus licheniformis</i> strain B	gram positive	150*
S10	AY241405	<i>Alteromonas alvinellae</i>	$\gamma$ -Proteobacteria	248*, 310*
S13	AY241408	<i>Shewanella alga</i>	$\gamma$ -Proteobacteria	206
S11	AY241406	<i>Vibrio fluvialis</i> (NCTC 11327 T)	$\gamma$ -Proteobacteria	206
S9	AY241404	<i>Vibrio furnissii</i> (ATCC 35016 T)	$\gamma$ -Proteobacteria	206
S2	AY241397	<i>Vibrio halioticoli</i> IAM14599	$\gamma$ -Proteobacteria	206
S1	AY241396	<i>Vibrio nereis</i> (ATCC 25917T)	$\gamma$ -Proteobacteria	206
S14	AY241409	<i>Vibrio</i> sp. BV25Ex	$\gamma$ -Proteobacteria	206
S7	AY241402	<i>Alpha proteobacterium</i> ISHR1	$\alpha$ -Proteobacteria	301*
S8	AY241403	Uncultured <i>Ruegeria</i> CtaxMed-2	$\alpha$ -Proteobacteria	301*
S15	AY241410	<i>Alpha proteobacterium</i> MBIC1876	$\alpha$ -Proteobacteria	302*
S17	AY241412	<i>Kocuria rhizophila</i> strain JPL-9	gram positive	302*
S16	AY241411	<i>Micrococcus kristinae</i>	gram positive	302*
S18	AY241413	<i>Micrococcus</i> sp. Wuba57	gram positive	302*
S5	AY241400	<i>Alteromonas alvinellae</i>	$\gamma$ -Proteobacteria	314*
S20	AY241415	<i>Pseudoalteromonas piscicida</i>	$\gamma$ -Proteobacteria	314*
S3	AY241398	<i>Tenacibaculum mesophilum</i>	CFB	368*
S19	AY241414	<i>Staphylococcus cohnii</i>	gram positive	371*
S12	AY241407	<i>Microbulbifer hydrolyticus</i>	$\gamma$ -Proteobacteria	422*

\* \*\* indicates TRF that could be matched with TRF derived from the sponge bacterial community.

**Table 4.** Possible identity of bacteria that could derive specific TRFs of interest based on the comparison from the RDP on-line analysis

Size of TRF (bp)		Bacterial strain	Accession no.	Phylogenetic branch	Reference			
TRF derived from both sponge and reference surfaces	302	<i>Abiotrophia</i>	AB022027	gram positive	[47]			
		<i>para-adiacens</i>						
		<i>Abiotrophia elegans</i>	AB022026	gram positive	[73]			
		<i>Enterococcus avium</i>	AF061008	gram positive	[15]			
		<i>Enterococcus dispar</i>	AF061007	gram positive	[18]			
		<i>Enterococcus casseliflavus</i>	AF039899	gram positive	[15]			
		<i>Enterococcus cecorum</i>	AF061009	gram positive	[21]			
		<i>Enterococcus faecium</i>	Y18294	gram positive	[74]			
		<i>Enterococcus faecalis</i>	Y18293	gram positive	[74]			
		<i>Enterococcus flavescens</i>	Y18295	gram positive	[63]			
		<i>Enterococcus gallinarum</i>	AF039898	gram positive	[15]			
		<i>Enterococcus hiraе</i>	Y17302	gram positive	[26]			
		<i>Enterococcus malodoratus</i>	Y18339	gram positive	[15]			
		<i>Enterococcus mundtii</i>	AF061013	gram positive	[16]			
		<i>Enterococcus raffinosus</i>	Y18296	gram positive	[17]			
		<i>Enterococcus saccharolyticus</i>	AF061004	gram positive	[68]			
		<i>Enterococcus sulfureus</i>	Y18341	gram positive	[58]			
		<i>Lactobacillus kefir</i>	AB024300	gram positive	[48]			
		<i>Lactobacillus lindneri</i>	X95422	gram positive	[5]			
		<i>Macrocooccus bovicus</i>	Y15714	gram positive	[51]			
		<i>Marinibacillus marinus</i>	AF530155	gram positive	[69]			
		<i>Tetragenococcus solitarius</i>	AF061010	gram positive	[24]			
			375	–	–	–	–	
		TRF derived exclusively from sponge surfaces	248	–	–	–	–	
				310	<i>Acidovorax anthurii</i>	AJ007013	β-Proteobacteria	[31]
					<i>Acidovorax avenae</i> subsp. <i>avenae</i>	AB021421	β-Proteobacteria	[4]
					<i>Acidovorax defluvii</i>	Y18616	β-Proteobacteria	[76]
					<i>Alcaligenes latus</i>	AB188125	β-Proteobacteria	[97]
					<i>Aquabacterium aqua1a</i>	AF089856	β-Proteobacteria	[46]
					<i>Aquabacterium</i> sp. <i>Aqua1b</i>	AF089857	β-Proteobacteria	[46]
<i>Aquaspirillum metamorphum</i>	Y18618				β-Proteobacteria	[76]		
<i>Burkholderia andropogonis</i>	AB021422				β-Proteobacteria	[4]		
<i>Burkholderia caribiensis</i>	Y17010				β-Proteobacteria	[2]		
<i>Burkholderia caryophylli</i>	AB021423				β-Proteobacteria	[4]		
<i>Burkholderia gladioli</i>	AB023647				β-Proteobacteria	[45]		
<i>Burkholderia glathei</i>	AB021374				β-Proteobacteria	[4]		
<i>Burkholderia graminis</i>	U96940				β-Proteobacteria	[87]		
<i>Burkholderia kururiensis</i>	AB024310				β-Proteobacteria	[98]		
<i>Burkholderia multivorans</i>	Y18703	β-Proteobacteria	[6]					



**Table 4.** Continued

Size of TRF (bp)	Bacterial strain	Accession no.	Phylogenetic branch	Reference
	<i>Burkholderia phenazinium</i>	AB021394	β-Proteobacteria	[4]
	<i>Burkholderia pseudomallei</i>	U91839	β-Proteobacteria	[8]
	<i>Burkholderia pyrrocinia</i>	AB021369	β-Proteobacteria	[4]
	<i>Comamonas terrigena</i>	AB021418	β-Proteobacteria	[4]
	<i>Cupriavidus necator</i>	AF027407	β-Proteobacteria	[86]
	<i>Curvibacter lanceolatus</i>	AB021390	β-Proteobacteria	[4]
	<i>Delftia acidovorans</i>	AB021417	β-Proteobacteria	[4]
	<i>Formivibrio citricus</i>	Y17602	β-Proteobacteria	[40]
	<i>Herbaspirillum huttiense</i>	AB021366	β-Proteobacteria	[4]
	<i>Herbaspirillum rubrisubalbicans</i>	AB021424	β-Proteobacteria	[4]
	<i>Hydrogenophaga flava</i>	AB021420	β-Proteobacteria	[4]
	<i>Magnetospirillum gryphiswaldense</i>	Y10109	α-Proteobacteria	[75]
	<i>Malikia spinosa</i>	AB021387	β-Proteobacteria	[4]
	<i>Matsuria chitosanitabida</i>	AB006851	β-Proteobacteria	[61]
	<i>Pseudomonas mephitica</i>	AB021388	γ-Proteobacteria	[4]
	<i>Pseudomonas saccharophila</i>	AB021407	γ-Proteobacteria	[4]
	<i>Ralstonia syzygii</i>	AB021403	β-Proteobacteria	[4]
	<i>Rhodiferax antarcticus</i>	AF084947	β-Proteobacteria	[56]
	<i>Roseateles depolymerans</i>	AB003626	β-Proteobacteria	[80]
	<i>Rubrivivax gelatinosus</i>	AB016167	β-Proteobacteria	[94]
	<i>Skermanella parooensis</i>	X90760	α-Proteobacteria	[20]
	<i>Variovorax paradoxus</i>	AB008000	β-Proteobacteria	[93]
	<i>Vogesella indigofera</i>	AB021385	β-Proteobacteria	[4]
311	<i>Brevundimonas alba</i>	AJ227785	α-Proteobacteria	[1]
	<i>Brevundimonas aurantiaca</i>	AJ227787	α-Proteobacteria	[1]
	<i>Brevundimonas bacteroides</i>	AJ227782	α-Proteobacteria	[1]
	<i>Brevundimonas diminuta</i>	D49422	α-Proteobacteria	[77]
	<i>Brevundimonas subvibrioides</i>	AB008393	α-Proteobacteria	[78]
	<i>Brevundimonas vesicularis</i>	AJ227781	α-Proteobacteria	[77]
	<i>Caulobacter henricii</i>	AB016846	α-Proteobacteria	[64]
	<i>Clostridium paraputrificum</i>	AB032556	gram positive	[25]
	<i>Thiocystis gelatinosa</i>	D50655	γ-Proteobacteria	[62]
	<i>Marichromatium purpuratum</i>	AF001581	γ-Proteobacteria	[42]
	<i>Nevskia ramosa</i>	AJ001011	γ-Proteobacteria	[33]
	<i>Paracoccus denitrificans</i>	Y16930	α-Proteobacteria	[67]

generated TRFs that could be matched with the TRFs derived from the sponge bacterial community samples (Table 3). With the exception of S10 (*Alteromonas alvinellae*), which generated two TRFs, all other bacterial isolates generated single TRFs that ranged from 129 to 422 bp upon *MspI* digestion of PCR-amplified 16S rRNA gene (Table 3). The TRF at 302 bp in the sponge bacterial community samples could match with the TRFs of four isolates that belonged to the gram-positive division (S16, S17, and S18) and the α-subdivision of Proteobacteria (S15; Table 3), whereas the TRF at 314 bp could match with the TRFs of two isolates that belonged to the γ-subdivision of Proteobacteria (S5 and S20). The TRF at 301 bp could match with those of another two isolates that belonged to the α-subdivision of Proteobacteria (S7 and S8). Other TRFs at 150, 248, 310, 368, 371, and 422 bp could match with the TRFs of one isolate belonging to a variety of taxa including the γ-subdivision

of Proteobacteria, the gram-positive group, and the Cytophaga-Flexibacter-Bacteroides group (Table 3). However, TRFs at 129 and 206 bp derived from the bacterial isolates were not observed in the TRFLP profiles of the sponge bacterial community samples.

Using the on-line analysis from the RDP [14], bacteria that could produce TRFs at particular size can be theoretically inferred. In most cases, one particular size of TRF could be generated from more than one sequence deposited in the database; for instance, about 200 sequences deposited in the database could theoretically generate TRF at 302 bp when digested with *MspI*. Sequences from all clones and some clinical strains were excluded from this study, and most of the strains listed in Table 4 came from environmental samples such as microbial mat, groundwater, deep-sea sediment, and marine- and freshwaters. After screening, 14 *Enterococcus* spp., 2 *Abiotrophia* spp., 2 *Lactobacillus* spp., and 3 other

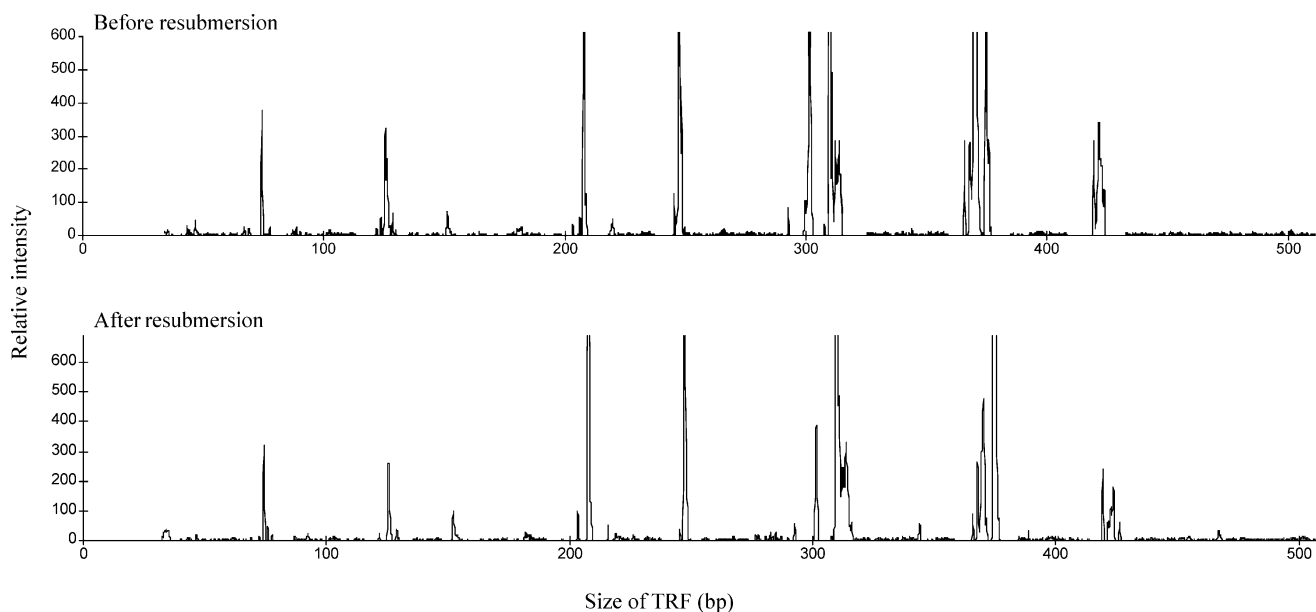
strains in the genera of *Macrococcus*, *Marinibacillus*, and *Tetragenococcus* could generate TRF of 302 bp (Table 4). Similarly, strains from 23 different genera, e.g., *Acidovorax*, *Burkholderia*, and *Herbaspirillum*, would result in TRF at 310 bp, whereas 6 *Brevundimonas* spp. and 6 other strains in the genera of *Caulobacter*, *Clostridium*, *Thiocystis*, *Marichromatium*, *Nevskia*, and *Paracoccus* could produce TRF at 311 bp (Table 4). However, no bacterial sequence deposited in the database could generate TRFs at 248 and 375 bp (Table 4).

## Discussion

Statistical analysis of TRF profiles revealed significant differences in genotype diversity of the bacterial communities between the reference surfaces and sponge surfaces at all sites and seasons (Fig. 4; reference surface vs sponge surface, Table 1). The diversity of the bacterial communities on the reference surface showed clear seasonality and site specificity, which were indicated by the segregation of the replicate bacterial communities derived from different sites and seasons in MDS ordinations (Fig. 4), and further supported by the significantly high *R* values in ANOSIM (sponge surface vs reference surface, Table 1). These site and seasonal differences of bacterial community structure could be a result of the changes in surface-related attributes, prevailing environmental conditions, and/or the availability of bacterial colonizers in the water column. One may question if the materials (made of polystyrene that is a rather inert, hydrophobic material) used for the

reference surface in this study could create arbitrary results. Based on our previous observations and the works in [88], the hydrophobicity/hydrophilicity of a surface may influence the composition of bacterial community at the initial stage of colonization, its effect often diminishes rapidly after the initial colonization, and the subsequent colonization by other bacteria is mainly influenced by environmental and biological factors. Therefore, we strongly believe that the observed seasonality and site specificity was a result of the differences of environmental conditions prevailing at each sampling site and time, including seawater temperature, the amount of terrestrial runoff, flow speed, turbidity, water chemistry, and nutrient content. All these factors, either singly or in combinations, can affect the composition of indigenous bacterial populations in the water column and thus the composition of bacterial communities that would eventually colonize the reference surface. It would be equally interesting to investigate the spatial and temporal variations of bacterial communities in the water columns to have a better understanding on how these variations influence the bacterial communities developed on the reference as well as on the sponge surfaces.

In contrast to the obvious seasonality and site specificity of the bacterial community on the reference surfaces, the bacterial communities on the sponge surfaces at individual sites or seasons were generally less distinguishable among different sites or seasons as shown by the interspersed MDS ordinations of the replicate bacterial communities in Fig. 4 and by the relatively low *R* values in ANOSIM (sponge surface vs sponge surface,



**Figure 5.** Representative electropherograms of TRFs derived from *MspI* digestion of PCR-amplified bacterial community DNA obtained from the sponge surfaces. Upper and lower panels show the bacterial communities before and after resubmersion, respectively, in a preliminary experiment.

Table 1). In contrast to the association with the inert reference surface, the bacterial community on the sponge surface may be influenced more by complex surface-related (i.e., sponge-related) attributes than that of environmental factors. These attributes may include the continuous release of bioactive metabolites (as chemical defense against bacterial epibiosis and/or the enrichment of specific bacterial types), the texture and topography of the sponge surface, and the presence of commensalistic meiofauna. The quantitative and qualitative differences in these attributes among individual sponges may explain the generally high variability of the bacterial community profiles derived from the sponge surface.

Although the bacterial communities on the sponge surfaces showed a certain degree of variability, eight TRFs in common were found among the three sites and were only found in the sponge-surface-associated bacterial communities (Table 2). Five of these TRFs (at 150, 247, 249, 419, and 420 bp) were found only in one or few sampling occasions; the sporadic occurrence of these TRFs suggests an occasional or less consistent association between the bacteria producing these TRFs and the sponge surface. On the other hand, the other three TRFs (at 248, 310, and 311 bp) were present consistently on the sponges at all sites and all seasons. The consistent association between the bacteria producing these three TRFs and the sponge surface may be the result of the selective forces (such as sponge's secondary metabolites) derived from the sponge or of the specific adaptation of these bacteria to the microenvironment on the sponge surface. In fact, many sponges may eliminate or nurture specific bacterial groups by the production of secondary metabolites [3, 10, 36, 37, 59]. When we compared TRFs of specific size with TRFs derived from the pure bacterial strains (Table 3) isolated from the surface of *M. adhaerens* [53], we deduced that the highly consistent TRFs at 248 and 310 bp may belong to *Alteromonas alvinellae* (S6). Crosby and Criddle [19] also pointed out that multiple TRFs could be derived from the same species because of ribosomal operon heterogeneity. On the other hand, the RDP on-line analysis indicated that the highly consistent TRFs at both 310 and 311 bp may also be derived from a variety of bacteria, but most of these bacteria were in the same genus. This may be caused by the possibility that closely related bacteria may share similar DNA sequence and have same restriction enzyme cutting site, thus generating TRFs of same length upon digestion by one restriction enzyme. The resolution of TRFLP analysis in differentiating different bacteria can be improved by multiple digests using several restriction enzymes individually followed by the use of TRFLP phylogenetic assignment tool [22, 50]. Although our data here cannot identify the bacterial species that are consistently associated with the sponge surface, our results suggested that the highly consistent and specific

bacterial TRFs observed on the sponge surface might be derived from a specific group of closely related bacteria. The highly consistent association of these bacteria with the sponge surface might reflect a beneficial interrelationship that enhances the survival of either one or both parties.

Two TRFs (at 302 and 375 bp) consistently appeared on both the sponges and reference surfaces at all sites and all seasons (Table 2). They were also found in the bacterial communities of the water columns of the three sites (data not shown). These TRFs may represent certain indigenous bacterial colonizers available in the water column throughout the year and colonized equally well on both the sponge and reference surfaces. Comparison of the TRFs to those derived from the bacteria isolated from ecologically relevant sites and to those estimated by the on-line analysis revealed that TRF at 302 bp might be derived from a number of bacteria in the gram-positive group (Tables 3 and 4). The inability of matching TRF at 375 bp to any bacterial isolates or sequences in the database might suggest that the bacteria deriving this particular size of TRF may not be culturable. It is also possible that small size discrepancies resulted from fragment sizing in analysis lead to the inability in matching. On the other hand, five other TRFs (at 301, 370, 371, 422, and 423 bp) that appeared on both surfaces only at particular seasons suggested a seasonal variation in the composition of indigenous bacterial colonizers.

Five TRFs (at 300, 301, 303, 370, and 422 bp) were exclusively associated with the reference surfaces at least at one sampling time (Table 2). However, unlikely the consistent association of some TRFs with the sponge surface, none of these five TRFs was present on the reference surface year round, suggesting possible fluctuations in bacterial communities with environmental factors.

The reference surface bacterial communities were developed on polystyrene dishes deployed in the close vicinity of the sponge for 7 days. One may also argue that the bacterial community developed under this condition may not be comparable to that on the sponge that has developed over months or years. This was not the case for our study for the following reasons. Before we decided to use 7 days as the submersion duration, we had already carried out a preliminary experiment, in which we had provided a "clean" sponge surface by removing its surface-associated bacteria and then resubmerged it for 7 days to allow the development of microbial film. We found that TRF patterns of the bacterial communities before and after cleaning and resubmersion were highly similar (similarity ~80%, Fig. 5), indicating that the development of bacterial film on the sponge surface could reach a "stable" stage after 7 days. Similarly, Qian *et al.* [66] showed that bacterial communities developed on

polystyrene dishes for 6, 9, and 12 days were highly similar, suggesting that a stable bacterial community could be obtained after 6 days of submersion in Hong Kong waters. Furthermore, it is almost impossible to obtain a "pure" microbial film after a longer time of submersion because other organisms such as invertebrate larvae will colonize the filmed surface. These organisms will carry their own bacteria added to the reference bacterial community and complicate the analysis of bacterial community originated from the reference dish only.

In summary, high degree of site and seasonal specificities in the reference bacterial communities demonstrated in this study might be attributed to the differences in environmental conditions. In contrast, the sponge-surface-associated bacterial communities were less distinguishable from each other but clearly differed from those on the reference ones, suggesting a rather distinctive and stable bacteria-sponge association irrespective of the environmental conditions. The consistent association of unique bacterial types with *M. adhaerens* over three geographically separate sites at four seasons provides direct evidence of a highly stable association between the two parties. Future works on 16S rDNA clone library of the surface-associated bacterial community will help us identify the unique bacterial associates of the sponge.

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