A bacterial culture-independent method to investigate chemically mediated control of bacterial epibiosis in marine invertebrates by using TRFLP analysis and natural bacterial populations

Tilmann Harder a, Stanley Chun Kwan Lau b, Wai-Yee Tam b, Pei-Yuan Qian b,*

a Institute for Chemistry and Biology of the Marine Environment, Carl von Ossietzky University, D-26111 Oldenburg, Germany
b Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, SAR Hong Kong, PR China

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Abstract

It has been postulated that a variety of physically undefended marine invertebrates have evolved strategies to control microbial epibiosis chemically. Ecologically meaningful experiments that demonstrate chemically mediated antibacterial effects are difficult due to the small number of cultivable bacteria. Based on the example of three sponges, this study introduces a culture-independent methodology to investigate chemically mediated control of bacterial epibiosis by analyzing the natural bacterial consortia. Organic extracts of sponges were immobi lized in hydrogels at tissue level concentrations and exposed to the same source of natural seawater for bacterial colonization. Terminal restriction fragment length polymorphism analysis of polymerase chain reaction-amplified bacterial community DNA obtained from these gels was shown to be a useful tool to study bacterial community shifts in response to sponge metabolites by comparing bacterial ribotypes obtained from the gel surfaces. Several terminal restriction fragments were absent relative to the control suggesting that settlement of specific bacteria was prevented. On the other hand, additional fragments occurred in some treatments, coinciding with higher bacterial abundance evidenced by DAPI counts of bacterial cells, indicating the bacterial utilization of sponge extract components. The advantages of this method are (1) a culture-independent approach, i.e. the assessment of antimicrobial activities against natural bacterial communities, (2) no restriction to particular modes of microbial colonization, i.e. antibiotic and repellent, and (3) the in situ assessment of antimicrobial compounds under flow conditions.

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Keywords: Microbial epibiosis; Marine invertebrate; Terminal restriction fragment length polymorphism; Chemical ecology

1. Introduction

Microorganisms and eukaryotic propagules (protists, diatoms and fungi) amount to millions per milliliter natural seawater in temperate geographic zones. Inundated substrata in the marine environment are therefore continuously exposed to ubiquitous colonizers. As a result, complex matrices comprised of bacteria, diatoms and fungi enmeshed in adsorbed organic debris colonize pristine marine surfaces within hours and develop into biofilm assemblages within days [1]. In the case of soft-bodied marine organisms (e.g. corals, sponges, and seaweeds), the extent of microbial colonization is influenced by the frequent renewal of epithelial layers [2,3] and host-specific components such as mucus, exudates, and secondary metabolites [4–8]. Bacterial epibiosis can be both beneficial and harmful to the host organism (basibiont). Whilst disease and tissue necrosis are obviously harmful [9], beneficial effects such as nurturing the host by nitrogen fixation or defense against pathogens are usually less obvious [10–12]. Because sedentary soft-bodied organisms generally lack means of mechanical defense, it has been postulated that they have evolved chemical mechanisms to regulate microbial epibiosis [13–15]. Host metabolites can potentially inhibit or reduce microbial epibiosis by repellent effects (negative chemotaxis) and inhibition of bacterial growth/attachment. A multitude of antibacterial compounds have been isolated from marine invertebrates [16] and identified in corals [17–20], ascidians [21] and sponges [22–25]. However, the potent activities of these compounds
against human pathogens should not be mistaken as ecologically relevant.

Naturally adapted control mechanisms of microbial epibiosis based on a chemical mode of action appeal to marine chemical ecologists. However, there is a great deal of uncertainty and bias when it comes to suitable experimental designs being able to demonstrate and verify such phenomena in an ecologically realistic fashion. The activities of natural antibiotics and microbial repellents have been conventionally investigated with the standard agar disc-diffusion assay [26] and a spectrophotometric chemotaxis conventionally utilized as substrates for bacterial attachment and colonization by natural bacterial populations. The bacterial community profiles on the gel surface were compared by the culture-independent, polymerase chain reaction (PCR)-based molecular fingerprinting technique of terminal restriction fragment length polymorphism analysis (TRFLP) [34,35]. The rationale for this approach was to overcome the disadvantages associated with a culture-dependent technique and moreover, to assess the overall community shifts in response to potential antimicrobial host allelochemicals.

2. Materials and methods

2.1. Collection of sponges and tissue extraction

Three species of sponges were collected by SCUBA divers at 5–10 m depth in a tropical reef south of Sanya, Hainan Province, China. These sponges were identified by N. de Voogd (Zoological Museum Amsterdam, ZMA) as Pachychalina sp. (Renieridae), Acanthella cavernosa (Dictyonellidae) and Xestospongia testudinaria (Petrosiidae) and voucher samples have been deposited at the ZMA under POR 17355 (X. testudinaria) and POR 17356 (A. cavernosa). The sponges were brought to the water surface in sealed plastic bags. The blot dry weight was measured on board and the tissue volume was determined by water displacement. A reference sample was stored in 96% ethanol for subsequent taxonomic identification. The chilled sponge tissue was taken to the Sanya Tropical Marine Ecology Station, South China Sea Institute of Oceanology, Chinese Academy of Science, and extracted twice in 1:1 dichloromethane:methanol for 8 h each with gentle agitation. The combined extracts were filtered through Whatman paper filter No. 1 and reduced by rotary evaporation to a concentration that was volumetrically equivalent to one tenth of the original sample tissue (i.e. 10× tissue level concentration).

2.2. Antibacterial assays

Antibacterial activities of sponge extracts were investigated by the standard paper disc-diffusion assay [26] on marine agar with replication (n = 3). Sponge extracts were pipetted onto circular paper discs (Whatman No. 1; disc volume = 20 mm³) to yield tissue level concentrations. For each extract, two experimental discs were incubated on a newly inoculated lawn of bacteria together with a negative control (organic solvent) and a positive control containing 10 μg of streptomycin. Assays were run at 28°C until bacterial growth was visible to the naked eye. The observed zones of growth inhibition between the disc and the bacterial lawn were measured to the nearest 0.5 mm. The bacterial community shifts in response to potential antimicrobial host allelochemicals.

2.3. Gel immobilization of tissue extracts

The immobilization of organic tissue extracts was adopted from [33] with slight modification. Briefly, known
volumes of sponge extract at 10× tissue level concentration were dried by rotary evaporation, leaving a semi-solid crude extract. These extracts were dissolved in the original volume of dimethylsulfoxide (DMSO) and transferred into sterile 50-ml Corning tubes. Gels were prepared by adding Phytagel® (Sigma Chemical) into a stirred beaker of boiling distilled water to yield a 4% (w/v) gel concentration. After the gel solution cooled to 70°C, the DMSO crude extracts were diluted to tissue level concentration with the liquid gel solution and vigorously mixed to evenly distribute the extract in the viscous hydrogel. Due to the high boiling point of DMSO (189°C) the extract volume remained constant during this procedure. A control was prepared accordingly with pure DMSO. The closed tubes were turned upside down and allowed to slowly solidify in a water bath at 60°C. After 5 min, air bubbles migrated out of the solution yielding a transparent gel cylinder. The cylinders were stored overnight at 4°C for a homogeneous diffusion of extract components through the gel. Subsequently, cylinders were removed and sliced into 5-mm-thick gel discs with sterile razor blades.

2.4. Development of bacterial communities on gel discs

The gel discs were pierced with fishing line and experimental treatments of each sponge and the control were anchored 10 cm below the water surface in individual 10-l aquaria (one vessel per treatment) continuously supplied with flow-through natural seawater directly from the field (one vessel avoided potential cross-contamination of experiments by water-borne extract components. The cylinders were stored overnight at 4°C for a homogenous diffusion of extract components through the gel. Subsequently, cylinders were removed and sliced into 5-mm-thick gel discs with sterile razor blades.

2.5. Bacterial counts and extraction of total bacterial community DNA

Bacteria on the gel surface were enumerated after visualization with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie, Switzerland). The formalin-fixed gel discs (n = 3) were rinsed with distilled water and stained with DAPI at a concentration of 0.5 µg ml⁻¹ for 5 min at room temperature. Gel discs were wet-mounted and bacterial cells were counted at 1000 × in 10 randomly chosen fields of view of known size under an epifluorescence microscope. In another set of experimental and control gel discs the area on both sides (6 cm²) was completely swabbed with sterile cotton buds for the collection of epibiotic bacteria. Swabs from each gel were individually suspended in 0.8 ml extraction buffer (100 mM Tris–HCl, 100 mM EDTA, 100 mM sodium phosphate, 1.5 M sodium chloride, 1% hexadecylmethylammonium bromide; at pH 8) in 2-ml microcentrifuge tubes. To lyse the cells, the samples were subjected to three cycles of freezing and thawing followed by 2 h incubation in 20% sodium dodecyl sulfate at 65°C. Cotton buds were removed and the total DNA was extracted with an equal volume of 24:1 chloroform:isoamyl alcohol followed by precipitation in isopropanol at room temperature for 15 min. The precipitated DNA was washed with cold ethanol and resuspended in 50 µl of autoclaved double-distilled water and frozen until use.

2.6. PCR

PCR of 16S rRNA genes (rDNA) in bacterial community DNA was performed in a total volume of 25 µl containing 1 µl of DNA template, 250 µM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotechnology, USA), 1 U of DNA Taq polymerase (Amersham Biosciences, USA) and 0.8 µM of each universal primer: 341F (5'-CCTAGGAGGCAGCAG-3') and 926R (5'-CCGATATTCTTTRAGTTT-3') [36, 37]. 926R was labeled at the 5' end with 6-carboxy fluorescein. PCR was performed at 95°C for 2 min; 15 touchdown cycles of 95°C for 30 s, 56°C for 3 min and 72°C for 3 min; the annealing temperature started at 56°C and was reduced to 40°C in increments of 1°C cycle⁻¹; 10 cycles of 95°C for 30 s, 40°C for 3 min and 72°C for 3 min; and 72°C for 10 min. Amplified DNA was verified by electrophoresis of 4 µl of PCR product in 1.5% agarose in 1× TAE buffer.

2.7. TRFLP analysis

Fluorescently labeled PCR products were purified with the Wizard® PCR prep DNA purification system (Promega, USA) according to the manufacturer’s protocol. Purified amplicons were digested with 20 U MspI (Boehringer Mannheim Biochemicals, USA) at 37°C for 6 h. Aliquots of digested products (10 µl) were mixed with 0.5 µl of internal size standard (ET550-R, Amersham Biosciences). This mixture was denatured for 2 min at 95°C and immediately chilled on ice before capillary electrophoresis on a MegaBACE® genetic analyzer (Amersham Biosciences) operated in Genotyping mode. After electrophoresis, the length of fluorescently labeled terminal restriction fragments (TRFs) was determined by comparison with internal standards by using the software ‘Fragment Profiler’ (Amersham Biosciences).

2.8. Statistical analysis

The patterns of TRFs of different bacterial community DNA samples were subject to non-metric multidimensional scaling (MDS). The Sorenson coefficient was calculated.
to produce a similarity matrix based on the total number of TRFs observed in all samples and the presence or absence of these TRFs in individual samples. The rank orders of similarity between different bacterial community DNA 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, with stress values smaller than 0.20 leading to an interpretable MDS pattern [38]. One-way analysis of similarities (ANOSIM) tested for global differences among individual samples. Pairwise comparisons tested for differences between samples when ANOSIM was significant. Calculations were performed with the PRIMER v3.1 computer program (Plymouth Marine Laboratory, UK). Bacterial abundances on experimental hydrogel surfaces were compared with a blank control by multiple pairwise comparison (Dunnett’s test) at the 95% confidence level. Statistical calculations were performed with the SPSS software package, Version 11.

3. Results and discussion

In this study, the extracts of the sponges Pachychalina sp., A. cavernosa and X. testudinaria were used for a comprehensive analysis of a chemical mechanism to control bacterial epibiosis. Organic tissue extracts were tested by the standard paper disc-diffusion assay on growth inhibition of 18 potential bacterial colonizers belonging to nine genera in three phylogenetic branches (α- and γ-Proteobacteria, Gram-positives; Table 1). We have repeatedly isolated these bacteria from artificial substrata submerged in subtropical waters at different locations and throughout different seasons. Therefore, these isolates were assumed to represent the cultivable omnipresent bacterial colonizers in this region. All the bacteria were susceptible to streptomycin to different extents, but not to the organic extraction solvent. The spectrum of susceptibilities towards the sponge extracts at tissue level concentration ranged from exclusive inhibition of γ-Proteobacteria by X. testudinaria to broad-spectrum inhibition of α- and γ-Proteobacteria as well as Gram-positive bacteria by Pachychalina sp. and A. cavernosa (Table 1). Some of the bacteria such as Rhodovulum sp., Ruergeria sp., Vibrio halioticoli, and Vibrio fluvialis were recalcitrant to all the extracts. These results suggested that despite their same origin and their presumptive exposure to the same set of bacterial colonizers in nature, the sponges under investigation might have evolved allelochemicals targeting different types of bacteria.

However, due to their isolation history via the same standard enrichment medium, the bacteria under investigation only represented an insignificant proportion of the total number of bacterial colonizers in the natural reef habitat and did not reflect the natural bacterial diversity. Therefore, a reliable assessment of the presumptive effects against microbial epibiosis was not warranted based on these data alone. To test whether the control of epibiotic bacteria by sponge metabolites could be examined with a natural community of bacterial colonizers in a culture-in-

![Table 1](https://academic.oup.com/femsec/article-abstract/47/1/93/510444)

<table>
<thead>
<tr>
<th>Bacterium (strain designation)</th>
<th>Growth inhibition next to disc (mm± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td>γ-Proteobacteria</td>
<td></td>
</tr>
<tr>
<td>Moraxella phenylpyruvica (UST000621-002)</td>
<td>–</td>
</tr>
<tr>
<td>Pseudovibrio piscida (UST0010260-005)</td>
<td>6.0 ± 2.6</td>
</tr>
<tr>
<td>Pseudovibrio sp. (UST0010723-006)</td>
<td>7.5 ± 2.1</td>
</tr>
<tr>
<td>Shevanella algae (UST010723-014)</td>
<td>–</td>
</tr>
<tr>
<td>Unidentified Vibrio sp. (UST950701-007)</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>Vibrio alginolyticus (UST98130-002)</td>
<td>6.7 ± 0.6</td>
</tr>
<tr>
<td>Vibrio fluvialis (UST010723-012)</td>
<td>–</td>
</tr>
<tr>
<td>Vibrio furnissi (UST010723-010)</td>
<td>–</td>
</tr>
<tr>
<td>Vibrio halotolerai (UST010723-002)</td>
<td>–</td>
</tr>
<tr>
<td>Vibrio harveyi (UST901209-010)</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>Vibrio hollisae (UST991130-052)</td>
<td>–</td>
</tr>
<tr>
<td>Vibrio vulnificus (UST001201-001)</td>
<td>–</td>
</tr>
<tr>
<td>α-Proteobacteria</td>
<td></td>
</tr>
<tr>
<td>Rhodovulum sp. (UST950701-012)</td>
<td>–</td>
</tr>
<tr>
<td>Roseobacter sp. (UST950701-009)</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>Ruegeria sp. (UST010723-008)</td>
<td>–</td>
</tr>
<tr>
<td>Gram-positive</td>
<td></td>
</tr>
<tr>
<td>Bacillus licheniformis (UST010723-004)</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Staphylococcus aureus (UST950701-005)</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Staphylococcus haemolyticus (UST950701-004)</td>
<td>–</td>
</tr>
</tbody>
</table>

Bacteria were not susceptible to a control of the pure organic solvent.
dependent approach, sponge tissue extracts were immobilized in a hydrogel matrix at tissue level concentration and exposed to natural seawater for the development of bacterial communities on the gel surface under conditions of water flow. Owing to this experimental setup, the treatments (sponge extracts) and controls were assumed to be exposed to the same pool of microbial colonizers, and bacterial community profiles on the hydrogel surface were supposed to be characteristically shaped by sponge-specific extract components.

Under the combination of PCR primers and the restriction enzyme used in this study, the number of discernible TRFs obtained from the treatments and the control ranged from 10 to 21 (Fig. 1). In total, 51 TRFs were used to create a similarity matrix based on Boolean character sets (1 or 0) corresponding to the presence or absence of a given TRF in a pattern. Due to the differential amplification of DNA fragments by PCR (i.e. PCR bias [39]), the signal intensity of TRFs did not reflect the actual abundance of the corresponding bacterial types and, therefore, was not considered in the analysis. The ANOSIM of TRFs of bacterial community DNA obtained from the hydrogel surfaces produced a global $R$ value at the significance level of 0.1%. The pairwise comparisons between different experimental groups resulted in the smallest possible level of significance between individual treatments and the control (Table 2). Two-dimensional scaling of TRFs of bacterial community DNA obtained from the hydrogel surfaces arranged the replicates of the same treatment in visually distinct groups distant from the control (Fig. 2). The stress value of 0.11 was a good indication of the confidence with which conclusions could be drawn from the MDS ordination.

The bacterial communities obtained from experimental gels were characterized by the absence and the additional presence of certain TRFs (i.e. ribotypes) in comparison to the control, which supposedly reflected a community of chemically unaffected bacterial colonizers (Fig. 1). In order to evaluate the potential of TRFLP analysis as a tool to assess a chemically mediated control of microbial epibiosis on Phytagei surfaces, it was the absence of ribotypes in comparison to the control which was of primary interest for this assessment. The absence of TRFs (e.g. at 250 bp in all the sponge metabolite-affected bacterial communities; at 150 bp in $A$. cavernosa, at 125 bp in $Pachychalina$ sp. and $X$. testudinaria) indicated that the corresponding types of bacteria were effectively inhibited by some extracts. However, the absence of a single TRF implied that multiple bacterial species might have been affected by sponge metabolites, because of the inability of TRFLP analysis to resolve bacterial communities at one TRF per bacterial species, regardless of the taxon [39,40].

The additional TRFs (e.g. from 200 to 225 bp in $Pachychalina$ sp. and $A$. cavernosa; Fig. 1) in combination with the absence in the control were considered to be specific to the sponge extracts. The additional TRFs were not observed in $Pachychalina$ sp. and $A$. cavernosa, which was indicative of the absence of corresponding bacterial types. The TRFs in $X$. testudinaria were assumed to be specific to the sponge extract, because the corresponding bacterial type was absent in the control.

Table 2

<table>
<thead>
<tr>
<th>Groups observed</th>
<th>$R$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$. cavernosa-Control</td>
<td>1.0*</td>
</tr>
<tr>
<td>$Pachychalina$ sp.-Control</td>
<td>0.759*</td>
</tr>
<tr>
<td>$X$. testudinaria-Control</td>
<td>0.87*</td>
</tr>
<tr>
<td>$Pachychalina$ sp.-$X$. testudinaria</td>
<td>0.278</td>
</tr>
<tr>
<td>$A$. cavernosa-$X$. testudinaria</td>
<td>0.926*</td>
</tr>
<tr>
<td>$A$. cavernosa-$Pachychalina$ sp.</td>
<td>0.778*</td>
</tr>
</tbody>
</table>

The pairwise comparison between groups is annotated with the $R$ value. The asterisk indicates statistical significance.

Fig. 1. Representative electropherograms of 5’ TRFs derived from MspI digestion of PCR-amplified bacterial community 16S rDNAs obtained from the surface of gel-immobilized extracts of the sponges $Pachychalina$ sp., $A$. cavernosa and $X$. testudinaria and a blank control without extract (Control).

Fig. 2. Two-dimensional MDS ordination of TRFs of bacterial community DNA samples obtained from three replicate surfaces of gel-immobilized extracts of the sponges $Pachychalina$ sp. (P), $A$. cavernosa (AC) and $X$. testudinaria (XT) and a blank control without extract (Control).
Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bacterial abundance (cells mm⁻²)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>68.958 ± 20.211</td>
<td></td>
</tr>
<tr>
<td>Pachychalina sp.</td>
<td>148.658 ± 35.553</td>
<td>0.026</td>
</tr>
<tr>
<td>A. cavernosa</td>
<td>189.748 ± 41.119</td>
<td>0.007</td>
</tr>
<tr>
<td>X. testudinaria</td>
<td>85.902 ± 4.057</td>
<td>0.613</td>
</tr>
</tbody>
</table>

The data shown are mean values of triplicate measurements with pseudo-replication (n=10) in each replicate. The bacterial surface abundance was statistically evaluated by multiple pairwise comparison against the control (Dunnett’s test).

with significantly higher bacterial abundances (Table 3) in these sponge metabolite-affected communities indicated that some extract components were utilized by certain bacteria and thus enhanced the growth/attachment of these bacteria. These observations were not surprising considering the potential nutritious value of the crude organic extracts for resistant heterotrophic bacteria. This enrichment effect confounded the assessment of overall community shifts in response to antibacterial extract components. Since chemical antibacterial effects could only be concluded from the absence of TRFs compared to the control, the potential out-competition of specific bacteria by the attracted heterotrophs did not allow a distinction between antibacterial effects of the host or specifically nurtured bacteria. Moreover, there was also the possibility of concluding false-negative results in the unlikely event that both the susceptible and the enriched bacteria produced identical TRFs.

Previously, the immobilization of organic tissue extracts in Phytagel® has been employed in antifouling assays with macroorganisms, such as marine invertebrate larvae [33]. It was also demonstrated that these gels exhibited excellent retention characteristics (e.g. 50% decrease of extract concentration within 21 days of exposure to seawater) and durability in long-term field experiments [33].

Methods for an effective, ecologically relevant evaluation of natural antimicrobial compounds continue to evolve. The methodology studied here may contribute to these developments by providing (1) a culture-independent approach to assess antimicrobial activities against natural bacterial communities, (2) no restriction to particular modes of microbial colonization, i.e. antibiotic and repellent, and (3) the in situ assessment of antimicrobial compounds under flow conditions. These characteristics may provide useful information to assess the potential of soft-bodied host organisms, such as sponges in this study, to chemically control bacterial epibiosis. However, the conceptual design of the immobilization of extracts at tissue level concentration rests on the assumption that antimicrobial host metabolites are evenly distributed throughout the host tissue and can be quantitatively extracted. Since these assumptions are based on an idealistic model, the uncertainty as to where a compound occurs in the tissue, how it is deployed, and thus what constitutes an ecologically realistic concentration to be assayed remains open given the present technology.

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