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A distinctive epibiotic bacterial community on the soft coral *Dendronephthya* sp. and antibacterial activity of coral tissue extracts suggest a chemical mechanism against bacterial epibiosis

Tilmann Harder, Stanley C.K. Lau, Sergey Dobretsov, Tsz K. Fang, Pei-Yuan Qian *

Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, SAR Hong Kong, PR China

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Abstract

Different bacterial community profiles were observed on the soft coral *Dendronephthya* sp. and an inanimate reference site using terminal restriction fragment length polymorphism analysis of bacterial community DNA. To correlate the observation with a chemical defense mechanism against bacterial epibiosis, antibacterial effects of coral tissue extracts and waterborne products of coral-associated bacterial isolates (11 morphotypes) were tested against indigenous benthic bacterial isolates (33 morphotypes) obtained in the vicinity of the coral colonies. The coral tissue extracts and waterborne products of coral-associated bacteria inhibited growth and attachment of indigenous bacterial isolates, suggesting an endogenous chemical and an exogenous biological mechanism against bacterial epibiosis in this soft coral.

Keywords: Marine bacteria; Soft coral; *Dendronephthya* sp.; Epibiosis; Terminal restriction fragment length polymorphism; Antimicrobial activity

1. Introduction

In the marine environment, animate and inanimate surfaces are rapidly covered with an organic layer as a result of molecular adsorption of dissolved organic matter, and colonization by bacteria, protozoa, algae and fungi [1,2]. Provided this association occurs between living organisms, this phenomenon is referred to as epibiosis. Bacterial epibiosis can cause a variety of beneficial effects to the basibiont, such as induction of macroalgal morphogenesis in the *Ulvales* class by symbiotic bacteria [3,4] or the interaction between macroalgae and nitrogen-fixing bacteria [5]. However, the adverse effects of bacterial epibiosis often outweigh the beneficial ones. For instance, soft-bodied marine invertebrates, such as sponges and soft corals, are susceptible to disease and tissue necrosis induced by bacteria [6,7]. Importantly, the adverse effects of bacterial epibiosis may reach beyond pathogenicity. Since bacterial films are important sources of chemical cues for larval settlement in many benthic marine invertebrates [8–10], bacterial epibiosis may promote subsequent colonization by macroorganisms. Epibiosis by macroorganisms in turn can significantly impair the basibionts ability to exchange gases and nutrients [11], damage the tissue by increased weight, rigidity and drag [12], and decrease the growth rate of photosynthetic basibionts by cutting surface irradiance levels [13,14].

Many soft-bodied marine invertebrates have evolved physical and chemical defense strategies to suppress epibiosis [11,15–19], e.g. sponges [20–22], ascidians [15], and soft corals [23–25] produce secondary metabolites that exhibit antibacterial and antialgal activities. While there is abundant evidence that crude organic extracts of marine invertebrates exhibit antimicrobial activity against medically important bacteria [26], the susceptibility of ecologically relevant bacteria to such activity has rarely been studied [17,24,27]. Besides bactericidal effects of the basibionts secondary metabolites, recent studies have increasingly indicated that some epibiotic bacteria evoke an inhibitory effect on the growth and attachment of co-occurring bacterial species competing for the same niche [28–32].

The soft coral *Dendronephthya* sp. is rarely colonized by sessile macro-organisms. Whilst a chemical defense mech-

* Corresponding author. Tel.: +852 2358 7331; Fax: +852 2338 1559.
E-mail address: boqianpy@ust.hk (P.-Y. Qian).

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anism against colonization by benthic invertebrates is known in *Dendronephthya* sp. [33,34], the ability of this coral to control bacterial epibiosis is poorly understood. In order to study whether there is some type of chemical defense to reduce bacterial epibiosis in *Dendronephthya* sp., the first objective of this study was to compare the profiles of established bacterial communities on the coral surface and an inanimate reference site by a molecular fingerprinting technique analyzing the terminal restriction fragment length polymorphism (TRFLP) in bacterial community DNA. Since the soft coral and the reference site were assumed to be exposed to the same pool of bacterial colonizers, differences in the bacterial community profile were hypothesized to stem from coral-related attributes. To investigate a potential chemical defense mechanism against bacterial epibiosis in *Dendronephthya* sp., both coral tissue extracts and waterborne products of coral-associated bacterial isolates were tested for antibacterial activities against omnipresent bacterial colonizers.

2. Materials and methods

2.1. Field collections

2.1.1. Corals

Soft corals were collected by SCUBA at 5–10 m depth in Port Shelter, Hong Kong (N 22′ 20″, E 114′ 20″). The rocky substratum around the corals' holdfasts was chisseled off without contacting the colonies. Corals were carefully brought to the water surface in sealed plastic bags and immediately flushed with autoclaved seawater to remove loosely attached bacteria. The volume of coral colonies was measured by water displacement. Colonies for solvent extraction were immediately frozen on dry ice and subsequently freeze-dried while those for the isolation of epibiotic bacteria and the extraction of community DNA from epibiotic bacteria were processed on site as described below.

2.1.2. Bacteria

Three coral colonies were individually swabbed with autoclaved cotton buds for the collection of epibiotic bacteria (hereafter referred to as coral-associated bacteria; CAB). Each colony was swabbed five times on different parts of the colony (the total swabbed area was ca. 18 cm^2). Swabs from the three colonies were combined and suspended in 15 ml of autoclaved, 0.22 μm-filtered seawater (FSW). Indigenous benthic bacteria were isolated from nine pre-sterilized glass slides that had been deployed for 7 days at a distance of 3 m around each sample colony (three slides for each colony). The natural biofilms (NBF) developed on these slides were swabbed accordingly (ca. 6 cm^2 on each slide), combined and suspended as described above. In the laboratory, bacterial suspensions were diluted (0.1 and 0.01×) in autoclaved FSW. A volume of 200 μl of each dilution was streaked onto nutrient agar (0.3% yeast extract, 0.5% peptone, 1.5% agar in FSW) with replication ($n = 3$) and incubated at 30°C under a 15:9 h light:dark photoperiod. After 1–3 days of incubation, bacterial colonies were examined under a dissecting microscope for conspicuous characteristics, such as color, shape, size, surface topography and the presence of granules. Distinguishable morphotypes were isolated, purified and subsequently stocked in 50% glycerol at −80°C.

2.1.3. Bacterial community DNA

The surfaces of two coral colonies were individually swabbed as described above (ca. 18 cm^2 on each colony). A reference bacterial community was swabbed from two rock pieces (ca. 9 cm^2 on each pieces) collected in the vicinity of each coral colony (ca. 3 m distance). Replicate swabs were combined and immediately frozen in 5 ml of lysis buffer (1% Triton X-100; 20 mM Tris–HCl at pH 8; 2 mM EDTA). In the laboratory, the bacterial samples were lysed at 95°C for 20 min. After centrifugation (10 000 rpm, 20 min), the total DNA in the supernatant was purified by phenol-chloroform extraction and subsequent precipitation in cold ethanol. The obtained DNA templates were dried in vacuo, resuspended in 20 μl of autoclaved double-distilled water and frozen until use.

2.2. Polymerase chain reaction (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 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotechnology, USA), 1 U of buffered AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA), 1.5 mM $MgCl_2$, and 1 μM of each universal primer: 355F-Hex (5′-ACTCCTACGGGAGGCAGC-3′) and 1055R (5′-CACGAGCTGACGACAGCCAT-3′) [35,36]. 335F-Hex was labeled at the 5′-end with the phosphoramidite dye 5-hexachlorofluorescein. PCR was performed at 95°C for 10 min; 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; and 72°C for 5 min. Amplified DNA was verified by electrophoresis of 5 μl of PCR mixtures in 1.5% agarose in 1× TBE buffer. The same protocol was applied for PCR of 16S rDNA obtained from individual bacterial isolates, except that non-labeled primers were used. DNA templates of bacterial isolates were obtained from pure colonies grown on nutrient agar according to Valsecchi et al. [37].

2.3. TRFLP analysis

Fluorescently labeled PCR products were purified with the Wizard® PCR preps DNA purification system (Promega, Madison, WI, USA) according to the manufacturer's protocol. Purified amplicons were digested with 20U *Msp*I

(Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) at 37°C for 6 h. Aliquots of digested products (2 μl) were mixed with 10 μl deionized formamide and 0.5 μl of internal size standard (ROX-500, Applied Biosystems, Foster City, CA, USA). This mixture was denatured for 5 min at 95°C and immediately chilled on ice before electrophoresis on an ABI PRISM™ 310 genetic analyzer (Applied Biosystems) operated in GeneScan mode. After electrophoresis, the length of fluorescently labeled terminal restriction fragments (TRFs) was determined by comparison with internal standards by using GeneScan software (Applied Biosystems).

2.4. DNA sequencing

Unlabeled PCR products were purified as described above and subject to cycle sequencing using the ABI PRISM™ big-dye terminator cycle-sequencing ready-reaction kit (Applied Biosystems) and the unlabeled 355F/1055R primer pair mentioned above. The DNA sequences were bidirectionally resolved on an ABI PRISM™ 310 genetic analyzer (Applied Biosystems) in sequencing mode.

2.5. Phylogenetic analysis

In order to determine the phylogenetic affiliation of the bacterial isolates their DNA sequences were compared with those in the non-redundant nucleotide database in GenBank using BLAST (Basic Local Alignment Search Tool). DNA sequences were aligned using the DNAtools package [38]. Phylograms were constructed by using the neighbor-joining algorithms.

2.6. Nucleotide sequence accession numbers

Obtained 16S rDNA sequences are available in the GenBank under the accession numbers AF465355–AF465398.

2.7. Coral extraction

The freeze-dry weight of 160 cm^3 of coral tissue was 26.6 g. The freeze-dried material was ground and sequentially extracted with four organic solvents of increasing polarity for 8 h each, i.e. 300 ml of hexane, chloroform, methanol and aqueous methanol (70%). The extracts were completely evaporated in tarred round-bottom flasks to determine crude extract dry-weights and subsequently redissolved in 5 ml of the respective organic solvent.

2.8. Enrichment of waterborne bacterial products

CAB were grown for 48 h in 30 ml of marine broth (0.5% peptone, 0.3% yeast extract (Oxoid) in FSW) at 30°C under a 15:9 h light:dark photoperiod. Bacteria were harvested from broth cultures by centrifugation (6000 × *g*, 15 min), washed and resuspended in 10 ml of autoclaved FSW. These suspensions were incubated for 12 h at room temperature to generate conditioned seawater (CSW) presumably enriched with waterborne bacterial products. Cell-free CSW was obtained after centrifugation and sterile-filtration (0.22 μm) of CSW.

2.9. Antibacterial activities

Antibacterial activities of different coral tissue extracts were investigated by the standard disc-susceptibility assay on marine agar with replication ($n = 3$) [39]. Coral extracts were redissolved in respective solvents and pipetted onto circular paper discs (Whatman No.1; disc volume = 28 mm^3) to yield a final extract dry weight of 1 mg per disc. For each extract, this amount represented a different multiple of the tissue level concentration, which was calculated by dividing the quotient of 1 mg extract dry weight to the disc volume of 28 mm^3 by the quotient of the coral extract freeze-dry weight to the coral volume. For each coral tissue extract, two experimental discs were incubated on a newly inoculated lawn of bacteria together with a negative organic solvent control and a positive control containing 10 μg of streptomycin. Assays were run at 30°C until bacteria developed a confluent film. In addition to the coral tissue extracts, waterborne products of CAB were tested analogously with FSW as a negative control. Paper discs were soaked once in CSW of respective bacterial suspensions, air-dried and directly employed in the disc-susceptibility assay. Observed zones of growth inhibition or halos between the disc and the bacterial lawn were measured to the nearest 0.5 mm.

2.10. Bacterial attachment assay

Inhibition of bacterial attachment on agar surface was assayed according to Wahl et al. [15] with modifications. Aliquots of coral extracts (5 μl) were evaporated on glass Petri dishes resulting in circular areas of dry extract residues (ca. 6 mm diameter). Pure solvents were applied accordingly and served as negative controls. Petri dishes were filled with seawater-based agar to form an even layer of 1 mm thickness. Following a 1-h incubation period, during which extract components diffused through the agar matrix, a bacterial suspension (ca. 10^8 cells ml^{-1}) was poured onto the agar. After 15 min, dishes were emptied and thoroughly dip-rinsed with sterile FSW. Attached bacteria were stained for 5 min with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole at a concentration of 0.5 μg ml^{-1}. The bacterial abundance was determined by epifluorescent microscopy at a magnification of 1250× in 10 randomly chosen fields of view (ZEISS Axiophot fluorescence microscope, $\lambda_{Ex} = 359$ nm, $\lambda_{Em} = 441$ nm). Inhibition of bacterial attachment was indicated by zones of lower cell densities over extract spots than control areas distant from sample spots or above solvent controls.

2.11. Statistical analysis

TRF patterns of different bacterial community DNA samples were subject to cluster analysis. The Jaccard coefficient was used 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. For the construction of a dendrogram, agglomerative clustering was performed using the similarity matrix and the unweighted pair-group average method. One-way ANOVA was used to analyze the results of bacterial attachment assays.

3. Results and discussion

Under the combination of PCR primers and restriction enzymes used in this study, cluster analysis of TRF patterns showed that the profiles of bacterial communities on the replicate coral colonies were highly similar, forming a cluster distantly related to the bacterial communities on the replicate reference surfaces (Figs. 1 and 2). The community profiles on the reference surfaces shared low similarity as evidenced by their occurrence in distantly related branches of the dendrogram (Fig. 2).

Since the soft coral and the reference surface were assumed to be exposed to the same pool of bacterial colonizers, the difference between the bacterial communities was hypothesized to result from coral-related attributes such as a chemical defense mechanism against bacterial epibiosis or the enrichment of specific bacterial types by coral products [42,43]. The high similarity between the coral surfaces was supposed to stem from the uniformity of these attributes. Contrary, the heterogeneity of indigenous bacterial community profiles on the reference surfaces was likely caused by qualitative differences of rock pieces (e.g. microtopography and mineral composition) and locally occurring events (e.g. grazing, dislocation and repositioning by external forces).

The comparatively high number of TRFs (i.e. ribotypes) in the reference samples suggested a more diverse bacterial community profile than in the coral samples. Although no distinctive TRFs were observed in the coral samples, the potential occurrence of coral-specific bacterial species cannot be eliminated since a given TRF can represent one or more bacterial species, regardless of the taxon [40,41]. De-

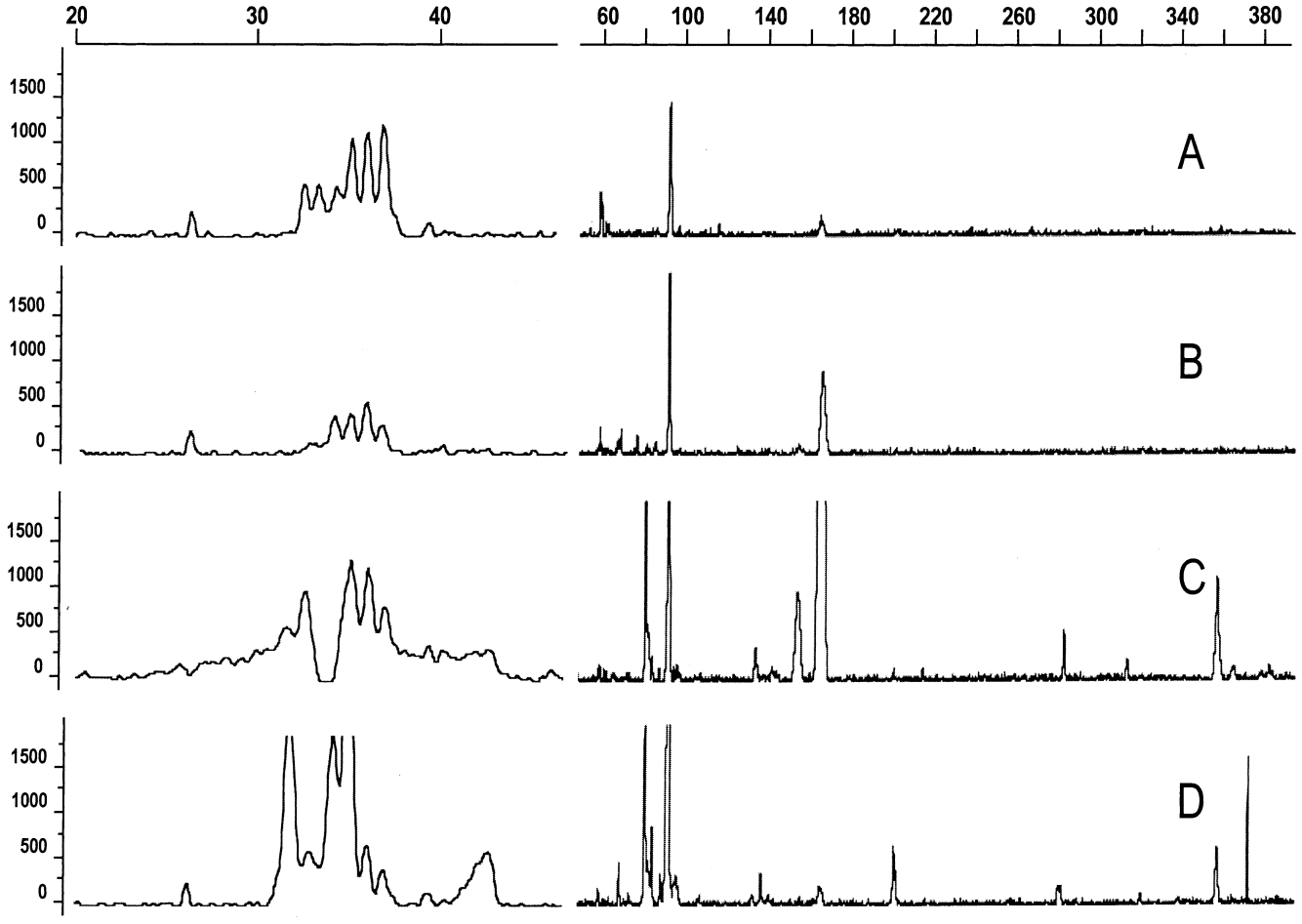

Fig. 1. Electropherograms of 5′-TRFs derived from *Msp*I digestion of PCR-amplified bacterial community 16S rDNAs obtained from coral (A,B) and rock surfaces (C,D). The 20–50 bp region is enlarged for clarity.

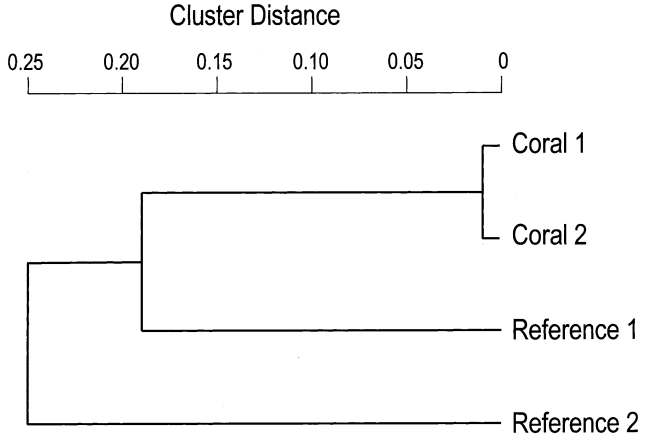

Fig. 2. Dendrogram showing the relatedness of bacterial communities on coral and rock surfaces. The dendrogram was constructed by using a similarity matrix determined by Jaccard coefficients and the unweighted pair-group average method.

spite the limitation of TRFLP analysis (including PCR bias) to reveal the complexity of native bacterial communities, this method provided sufficient experimental evidence to demonstrate a difference between the bacterial communities on the coral and the reference surface.

In order to analyze the observed difference regarding a potential chemical defense mechanism against bacterial epibiosis, coral tissue extracts and waterborne products of CAB were tested for antibacterial activities against indigenous benthic bacterial isolates. Contrary to the usage of a rather established biofilm on the reference site 'rock' for TRFLP, a young biofilm was chosen for the isolation of indigenous benthic bacteria. By utilizing bacteria from a comparatively young film, we intended to study the effect of coral metabolites on early-successional bacteria, which were assumed to be omnipresent colonizers of the coral.

Altogether, 33 morphotypes of indigenous benthic bacteria were obtained. Among those, comparative analysis of

Table 1
Phylogenetic affiliation of bacterial isolates from the reference site

Isolate	Strain designation	Closest match in GenBank		
		Strain	GenBank accession no.	Sequence similarity (%)
γ-Proteobacteria				
NBF-34	UST991130-034	*Vibrio* sp. AB003	AF369642	98.3
NBF-16	UST991130-016	*Vibrio* sp. AS-42	AJ391203	96.9
NBF-19	UST991130-019	*Vibrio* sp. BB4	AF319768	99.5
NBF-31	UST991130-031	*Vibrio* sp. BB4	AF319768	98.6
NBF-10	UST991130-010	*Vibrio* sp. BV25Ex	AF319769	98.9
NBF-17	UST991130-017	*Vibrio* sp. BV25Ex	AF319769	98.9
NBF-11	UST991130-011	*V. campbellii*	AY035896	99.5
NBF-20	UST991130-020	*V. campbellii*	AY035896	98.2
NBF-22	UST991130-022	*V. campbellii*	AY035896	99.0
NBF-33	UST991130-033	*V. diabolus*	X99762	97.8
NBF-18	UST991130-018	*V. ichthyoenteri* DSM 14397T	AJ421445	96.9
NBF-24	UST991130-024	*V. nereis* ATCC 25917T	X74716	98.0
NBF-28	UST991130-028	*V. nereis* ATCC 25917T	X74716	96.0
NBF-13	UST991130-013	*V. parahaemolyticus* clone Vp16	AF388387	96.2
NBF-9	UST991130-009	*V. pectenicida*	Y13830	96.6
NBF-14	UST991130-014	*V. pectenicida*	Y13830	98.6
NBF-25	UST991130-025	*V. pectenicida*	Y13830	98.1
NBF-29	UST991130-029	*V. pectenicida*	Y13830	97.9
NBF-7	UST991130-007	*V. tubiashi* ATCC 19109T	X74725	97.7
NBF-23	UST991130-023	*V. tubiashi* ATCC 19109T	X74725	96.5
NBF-12	UST991130-012	*Photobacterium* sp. HAR72	AB038032	96.6
NBF-5	UST991130-005	*Photobacterium leiognathi*	D25309	96.4
NBF-4	UST991130-004	*Pseudoalteromonas* sp. S9	U80834	99.1
NBF-8	UST991130-008	*Pseudoalteromonas* sp. S9	U80834	98.2
NBF-30	UST991130-030	*Pseudoalteromonas haloplanktis* subsp. *tetraodonis*	AF214729	98.9
NBF-27	UST991130-027	Marine bacterium PP-145.98	AJ296157	98.4
NBF-1	UST991130-001	Uncultured marine bacterium HstpL18	AF159668	97.4
NBF-6	UST991130-006	Uncultured marine eubacterium HstpL94	AF159671	98.4
NBF-3	UST991130-003	Unidentified *γ-Proteobacterium* HTC036	AB010855	98.9
Cytophaga-Flexibacter-Bacteroides				
NBF-35	UST991130-035	*Tenacibaculum mesophilum* MCBI4357	AB032504	93.7
NBF-36	UST991130-036	*T. mesophilum* MCBI1543	AB032502	96.8
Gram-positive				
NBF-26	UST991130-026	Unidentified bacterium 61610	AF227839	98.6
NBF-32	UST991130-032	*Bacillus* sp. YSS/2001-3	AF417874	98.1

The 16S rRNA gene sequences of individual bacterial isolates were compared to the nucleotide sequences in GenBank. The closest matching nucleotide sequence for each bacterial isolate is indicated by the strain name and accession number.

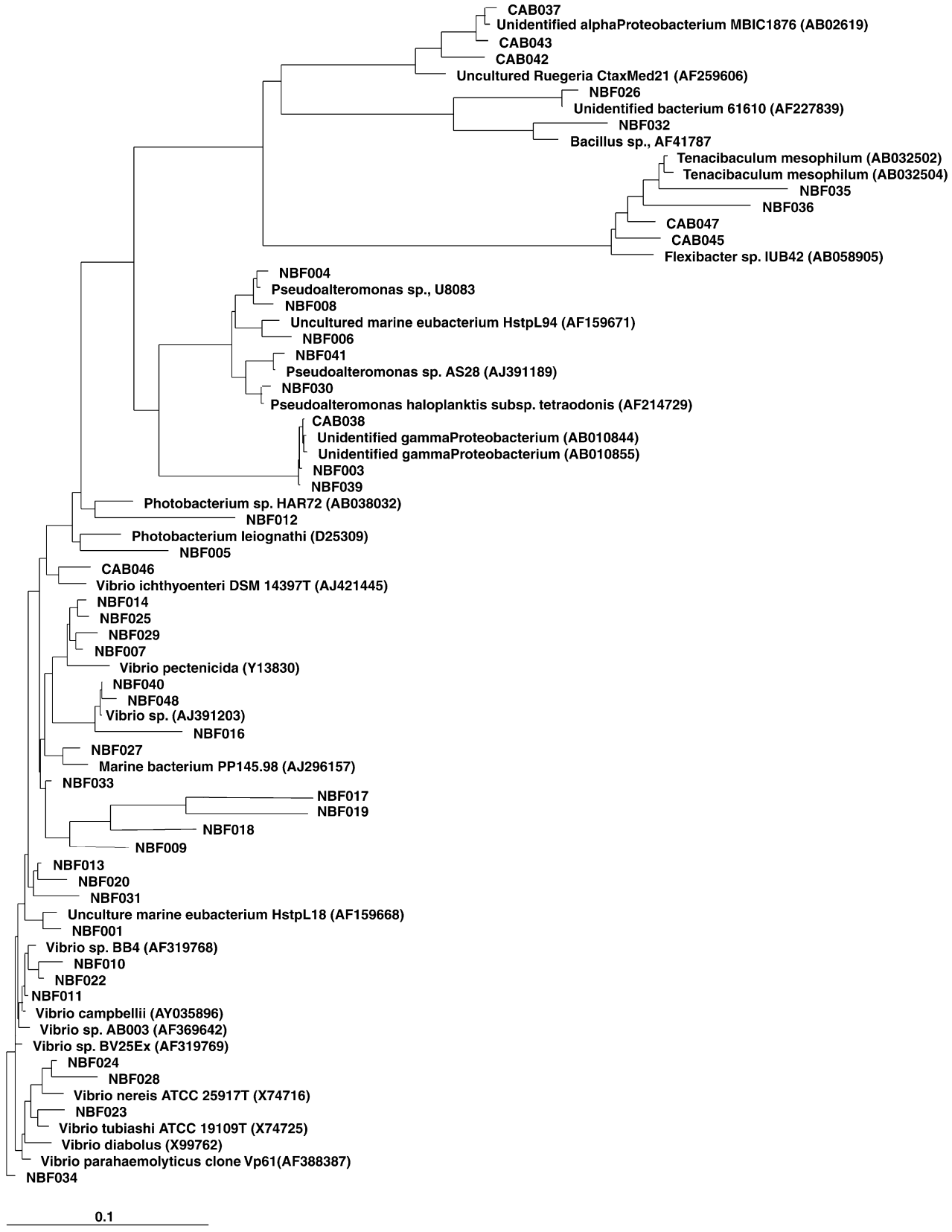

Fig. 3. Neighbor-joining phylogenetic tree showing the genetic distances between CAB, indigenous benthic bacterial isolates (NBF) and their closest relatives. The nucleotide sequence accession numbers of the closest matching strains are indicated in parentheses. The scale bar represents 0.1 substitution per nucleotide position.

Table 2
Phylogenetic affiliation of bacterial isolates from coral surface

Isolate	Strain designation	Closest match at the GenBank		
		Strain	GenBank accession no.	Sequence similarity (%)
γ-Proteobacteria				
CAB-41	UST991130-041	*Pseudoalteromonas* sp. AS-28	AJ391189	97.8
CAB-40	UST991130-040	*Vibrio* sp. AS-42	AJ391203	99.3
CAB-48	UST991130-048	*Vibrio* sp. AS-42	AJ391203	98.4
CAB-46	UST991130-046	*V. ichthyoenteri* DSM 14397T	AJ421445	97.5
CAB-38	UST991130-038	Unidentified *γ-Proteobacterium* HTC036	AB010855	98.7
CAB-39	UST991130-039	Unidentified *γ-Proteobacterium* HTC34	AB010844	99.4
α-Proteobacteria				
CAB-37	UST991130-037	Unidentified *α-Proteobacterium* MBIC1876	AB026194	99.5
CAB-43	UST991130-043	Unidentified *α-Proteobacterium* MBIC1876	AB026194	98.7
CAB-42	UST991130-042	Uncultured *Ruegeria* CtaxMed-21	AF259606	95.5
Cytophaga-Flexibacter-Bacteroides				
CAB-45	UST991130-045	*Flexibacter* sp. IUB42	AB058905	96.6
CAB-47	UST991130-047	*Tenacibaculum mesophilum*	AB032504	98.4

The 16S rRNA gene sequences of individual bacterial isolates were compared to the nucleotide sequences in GenBank. The closest matching nucleotide sequence for each bacterial isolate is indicated by the strain name and accession number.

16S rRNA gene sequences revealed 23 phylotypes affiliated to five genera (*Bacillus*, *Photobacterium*, *Pseudoalteromonas*, *Tenacibaculum* and *Vibrio*) in three phylogenetic branches (*γ-Proteobacteria*, *Cytophaga-Flexibacter-Bacteroides* and Gram-positive) (Table 1, Fig. 3). Eleven bacterial morphotypes (yielding nine individual phylotypes) were isolated from the coral surface. They belonged to five genera (*Flexibacter*, *Pseudoalteromonas*, *Ruegeria*, *Tenacibaculum* and *Vibrio*) in three phylogenetic branches (*Cytophaga-Flexibacter-Bacteroides*, *α-* and *γ-Proteobacteria*) (Table 2; Fig. 3). Although some indigenous benthic isolates were affiliated to the same phylotype, the neighbor-joining phylogenetic tree and cross-comparison of nucleotide sequences indicated an appreciable genetic variability among them (e.g. NBF-7 and -23; NBF-24 and -28; Table 3; Fig. 3). A similar observation was made for some of the CAB and indigenous benthic isolates that were affiliated to the same phylotype (e.g. NBF-16, CAB-40 and -48, NBF-35 and CAB-47; Table 3, Fig. 3). In contrast to the predominance of *γ-Proteobacteria* among the culturable indigenous benthic bacteria (91% of isolates), this taxon accounted for only about half of the CAB (55%). In addition to the results obtained by TRFLP analysis, the exclusive association of *α-Proteobacteria* with the coral indicated a distinctive bacterial community profile on the coral.

The coral tissue extracts inhibited the growth of indigenous benthic isolates at different levels. With the exception of four isolates (NBF-1, -3, -26 and -34), either complete inhibition of growth or distinct halos were observed around experimental discs (Fig. 4A). Halos were clearly distinguishable from the unaffected bacterial lawn by reduced opacity or the absence of trapped gas-vesicles in the agar matrix. While the former may indicate a weak inhibition of bacterial growth, the latter suggested an altered production of volatile metabolites. The susceptibility of

Table 3
Cross-comparison of 16S rRNA gene sequences among isolates having the same closest matching strain at the GenBank

Isolates	Closest matching strain at the GenBank	Similarity (%)
NBF-3, CAB-38	Unidentified *γ-Proteobacterium* HTC036	98.5
NBF-16, CAB-40	*Vibrio* sp. AS-42	96.6
NBF-16, CAB-48	*Vibrio* sp. AS-42	95.9
NBF-18, CAB-46	*V. ichthyoenteri* DSM 14397T	95.9
NBF-35, CAB-47	*Tenacibaculum mesophilum* MCBI4357	94.1
CAB-37, CAB-43	Unidentified *α-Proteobacterium* MBIC1876	98.7
CAB-40, CAB-48	*Vibrio* sp. AS-42	99.1
NBF-4, NBF-8	*Pseudoalteromonas* sp. S9	98.5
NBF-7, NBF-23	*V. tubiashi* ATCC 19109T	96.0
NBF-9, NBF-14, NBF-25, NBF-29	*V. pectenicida*	97.0–98.9
NBF-10, NBF-17	*Vibrio* sp. BV25Ex	98.2
NBF-11, NBF-20, NBF-22	*V. campbellii*	97.7–98.6
NBF-19, NBF-31	*Vibrio* sp. BB4	99.0
NBF-24, NBF-28	*V. nereis* ATCC 25917T	97.3

The percentage similarities shown were obtained by using BLAST (Basic Local Alignment Search Tool).

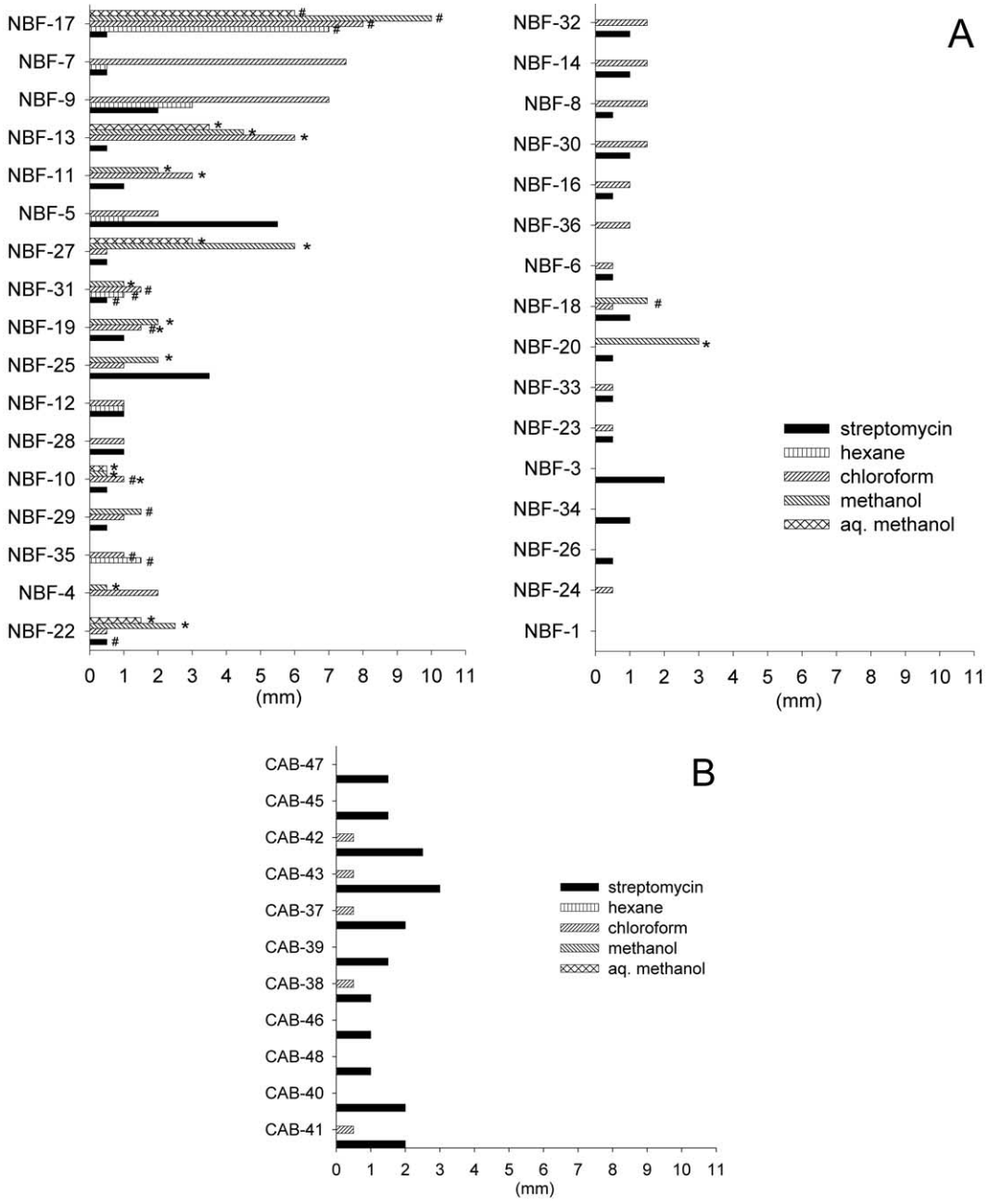

Fig. 4. A: Susceptibility of 33 indigenous benthic bacteria to four organic solvent extracts of coral tissue (1 mg extract dry weight per disc) and a positive control of streptomycin (10 μg per disc). B: Susceptibility of 11 CAB to four organic solvent extracts of coral tissue (1 mg extract dry weight per disc) and a positive control of streptomycin (10 μg per disc). Bars represent mean distances of bacterial growth inhibition between the disc and the unaffected bacterial lawn or other distinctive characteristics, such as reduced opacity (#) or the absence of trapped gas-vesicles in the agar matrix (*). Bacteria were not susceptible to the solvent controls.

bacteria to coral extracts comprising a wide polarity range of organic solvents indicated that more than one active compound was separated from coral tissue by sequential extraction. Some of the apparently closely affiliated indigenous benthic isolates displayed different susceptibility to the coral extracts (e.g. NBF-11 and -22; NBF-14 and -25), indicating possible phenotypic differences among these isolates. Similar observations were also made for the isolates' ability to attach to agar (NBF-4 and -8; Fig. 5) and their susceptibility to CAB metabolites (NBF-14 and -25; Fig. 6).

The disc-susceptibility assays were performed with equal dry-weights of individual coral extracts on paper discs. Considering that coral metabolites obtained by sequential extraction were disproportionally enriched in solvents of different polarity, the individual extracts were assayed at different multiples of the 'tissue level concentration', a measure frequently used to describe a presumptive homog-

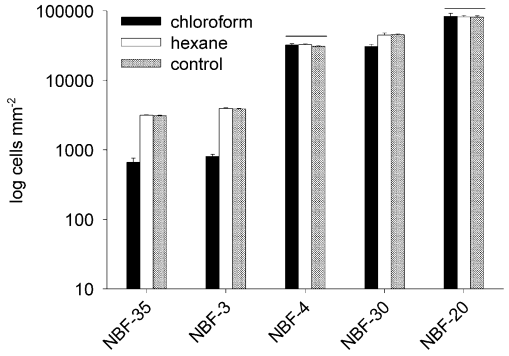

Fig. 5. Inhibition of bacterial attachment of selected indigenous benthic bacteria by chloroform and hexane extracts of coral tissue versus a negative control (pure solvent). Bars represent mean cell densities ± S.D. Lines above bars indicate groups among which the means did not differ significantly (one-way ANOVA, $\alpha = 0.05$).

enous distribution of extractable compounds in the tissues of soft-bodied organisms [18,23]. Based on the dry-weights of individual tissue extracts, the hexane, chloroform, methanol, and aqueous methanol fraction of extractable coral metabolites were assayed at 21×, 16×, 2× and 1× tissue level concentration, respectively. The extracts of medium polarity, i.e. methanol and chloroform, affected 40% and 85% of bacteria, respectively, while the extracts of high (aqueous methanol) and low (hexane) polarity affected 15% and 21% of bacteria, respectively. These results indicated that the unpolar fraction of extractable coral metabolites was not a major contributor for growth inhibition of indigenous benthic bacterial isolates.

Besides the effect of coral tissue extracts on growth inhibition of indigenous benthic isolates, their impact on another aspect of the bacterial colonization process, i.e. attachment, was evaluated. This assessment was limited to a small number of isolates and to unpolar coral tissue extracts due to the lack of attachment to an agar surface of most isolates under investigation and due to the high solubility of polar tissue extract components in the unpolymerized agar matrix. Three isolates (NBF-35, -30 and -3) were significantly inhibited by the chloroform extract, two of which (NBF-35 and -30) were not only affected by inhibition of attachment (Fig. 5), but also by inhibition of growth (Fig. 4A). Thus, with the relatively small selection of culturable bacteria, our results suggested that coral-derived compounds may exhibit different modes of action to suppress bacterial colonization.

Contrary to the indigenous benthic bacteria only half of the CAB were affected by the coral extracts. This observation indicated that some of the CAB may have bypassed the chemical defense or are more tolerant to coral metabolites and may have led to a symbiotic relationship between certain CAB and the soft coral. From the perspective of the host organism, the functional role of this relationship might be the acquisition of an exogenous defense mechanism against epibiosis by harmful bacteria. This phenomenon is based on the fact that bacteria may exert antibacterial activity as a selective advantage when in competition with other bacteria colonizing the same niche [28–31]. To investigate this possibility in *Dendronephthya* sp., waterborne products of CAB were subject to disc-susceptibility assays. Seven benthic indigenous isolates were susceptible to waterborne products of three CAB (Fig. 6). In addition to the antibacterial effect of coral-derived compounds, this result suggested an exogenous defense mechanism against bacterial epibiosis.

In summary, our study demonstrated that compounds derived from coral colonies and some CAB suppressed ecologically relevant bacteria by two modes of action, i.e. inhibition of growth and attachment. To infer ecological significance of the laboratory results obtained in this study, the test concentrations of coral tissue extracts were required to be ecologically realistic. Since not only the chemical nature of biologically active coral metabolites but also their distribution within the coral tissue were unknown, attempts to mimic ecological realism were prone to uncertainty or bias. Besides this ambiguity, the bioassays confounded a comparison with the field situation since diffusion rates of coral metabolites through the agar matrix, and hence zones of growth inhibition, may vary significantly with respect to the polarity of these compounds. Considering the ambiguities of ecological realistic test concentrations, at this stage it can only be suggested that our results explain the observed difference in bacterial community profiles on *Dendronephthya* sp. and the inanimate reference surface. More specific accounts on the control of bacterial epibiosis by the coral necessitate not only the identification of bioactive metabolites but also mea-

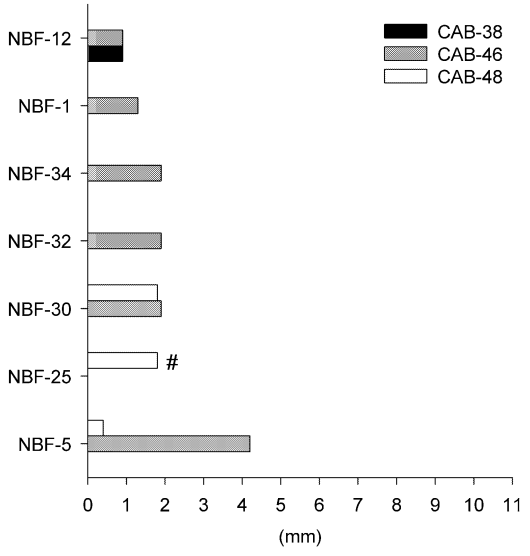

Fig. 6. Susceptibility of seven indigenous benthic bacterial isolates to waterborne products of three bacterial epibionts of the coral (black, gray and white bars). Bars represent mean distances of bacterial growth inhibition between the disc and the unaffected bacterial lawn or distinctively reduced opacity of the colony (#). Bacteria were not susceptible to the FSW control.

surements of their concentrations in the epithelial coral tissue and natural release rates in the field.

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