Antagonistic antimicrobial activity of marine fungi and bacteria isolated from marine biofilm and seawaters of Hong Kong

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ABSTRACT: The antagonistic interaction between marine fungi and marine bacteria was investigated using 19 bacterial species isolated from marine biofilm and 46 waterborne fungal isolates obtained from Hong Kong waters. Of the 46 fungal isolates, 70% inhibited the growth of at least 1 target bacterial species. Active fungal strains belonged mainly to the genera *Cladosporium*, *Paraphaeosphaeria*, *Trichoderma*, *Alternaria*, *Phoma*, and *Arthrinium*. Eight fungal isolates (17% of all fungal isolates tested) were sensitive to at least 1 target bacterial species. Eleven of the 19 bacterial species showed antifungal activity. Two *Staphylococcus* strains inhibited the growth of all of the sensitive fungal strains. The results indicated that marine fungi could provide an important source of natural antimicrofouling compounds and that the interaction between marine fungi and marine bacteria in biofilms cannot easily be generalized.

KEY WORDS: Marine fungi · Marine bacteria · Microbial interaction · Microfouling

INTRODUCTION

All surfaces, both animate and inanimate, submerged in seawater become covered with a thin layer of biofilm made of biomolecules, bacteria, fungi, protozoa and diatoms (Clare et al. 1992). This layer is highly complex, dynamic and super rich in molecular signals produced by marine bacteria and diatoms, which can directly control larval settlement and the subsequent growth of marine invertebrates (Harder et al. 2002, Lam et al. 2003, Qian et al. 2003). Since the bacteria in biofilm are important sources of molecular signals, they play a regulatory role in the recruitment patterns and population dynamics of marine organisms.

It has been illustrated that many physical and chemical factors, such as hydrodynamic conditions, the intercellular signal, and metabolic interactions among different cells and species of bacteria, can affect the development of biofilms; thus, the molecular signals for larval settlement produced by a biofilm can change substantially (Wieczorek et al. 1996, Davey & O'Toole 2000). Interaction among different film components such as marine fungi and bacteria will also undoubtedly have direct effects on the chemical signals produced by the film. Since marine fungi have been identified as major corrosive organisms of man-made marine installations and as pathogens of many marine plants and animals (Kohlmeyer & Kohlmeyer 1979), much research has focused on the growth of corrosive marine fungi (Bettucci et al. 1999) and the interaction between fungal pathogens and their hosts (Dube et al. 2002). However, the interaction between marine fungi and bacteria in biofilms as well as the role of marine fungi in the development of marine biofilm have hardly been studied, although marine-derived fungi have been shown to produce interesting bioactive metabolites (Bernan et al. 1997, Pietra 1997), including some potential antibiotics (Strongman et al. 1987, Cheng et al. 1994). In comparison, substantial amounts of work have been done on the interaction between terrestrial fungi and bacteria, and this has led to numerous discoveries, including the discovery and

application of penicillin. Therefore, studying the cross-kingdom interaction between marine fungi and bacteria could be of interest for both the applied and pure sciences.

In contrast, since marine biofouling is extremely widespread and causes serious operational problems and huge economic loss (Evans & Clarkson 1993), many potential antifouling compounds have been isolated from sponges (Martin & Uriz 1993, Kelman et al. 2001, Kubanek et al. 2002), corals (Michalek & Bowden 1997, Kelman et al. 1998), algae (Mtolera & Semesi 1996), ascidians (Wahl et al. 1994), seagrasses (Jensen et al. 1998), sea stars (Iken et al. 2000), and sea pansies (Rittschof et al. 1986). Microorganisms associated with some of these macroorganisms, however, have been suggested as the true source of their antifouling protection (Jensen & Fenical 1994, Kon-Ya et al. 1995, Bernan et al. 1997), making bioactive substances from marine microorganisms a new research frontier of natural product chemistry. Although marine fungi have been systemically screened for their antibacterial activity (Cuomo et al. 1995, Christophersen et al. 1999), screening for the antibacterial activity against biofilm bacteria (microfoulers) has been scarce. Since microbial fouling is often a prerequisite for the development of a macrofouling community, the control of microbial fouling may help to control macrofouling.

The aims of this study were (1) to screen for the antibacterial activity of marine fungi isolated from Hong Kong waters against 19 fouling bacteria, and (2) to investigate the possible antagonistic interaction between these fungi and bacteria.

MATERIALS AND METHODS

Isolation of marine fungi from Hong Kong waters. Seawater from the mangrove stand at Kei Ling Ha Lo Wai, Sai Kung (114° 21' E, 22° 24' N), was collected in November 2002 using sterile falcon tubes. The samples were diluted 10- and 100-fold with autoclaved filtered (0.22 mm) seawater (FSW). Aliquots of 200 ml from each water sample were spread onto nutrient agar plates (containing 2% glucose, 1% peptone, 2% agar and FSW). The antibiotics streptomycin and penicillin at final concentrations of 100 and 50 mg l^{-1}, respectively, were added to each agar plate to inhibit the growth of bacteria. Two replicate agar plates were used for each sample. After 2 d incubation at 27°C, the inoculated agar plates were examined daily using a dissecting microscope at 20× magnification for the presence of developing fungal hyphae. Distinct fungal colonies on the agar plates were then transferred to new agar plates for further isolation and purification.

Identification of the fungal isolates. *Morphological examination:* The pure fungal isolates were maintained on corn meal agar (Oxoid) until sporulation occurred. The spore-producing structures were dissected and removed from the agar surface with a sterile dissecting needle for microscopic examination, and subsequently mounted in glycerol. The microscopic features were then observed using a Leica compound microscope at 40× magnification with bright-field illumination. All isolates were identified to the genus level, based on the morphology of the sporulating structures as well as the morphology and pigmentation of the conidia and conidiophores, following the saccardo classification system (Barnett & Hunter 1987).

Molecular examination: A molecular approach was adopted to identify non-sporulating strains. These fungal isolates were inoculated into a glucose peptone broth medium (containing 2% glucose and 1% peptone) and incubated at 27°C on a shaker set at 120 rpm for 1 wk. The mycelia were then filtered through 8 layers of sterile cheesecloth and washed with autoclaved double distilled H$_2$O. DNA extraction was performed using the CTAB extraction method (Zhou et al. 1996). The ITS (internal transcribed space) flanking the 5.8S region of the ribosomal DNA gene was amplified. One pair of primers, ITS5 and ITS4 (White et al. 1990), were used to amplify the ITS1 and ITS2 regions of the rDNA gene cluster. Each PCR mixture contained 1 U of AmpliTaq Gold™ DNA polymerase (Applied Biosystems), GeneAmp® PCR buffer (Applied Biosystems), 2 µM of each primer, 5 mM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (Pharmacia Biotechnology), and 1 µl of crude DNA extract in a total volume of 25 µl. The PCR was performed on a PTC-100™ programmable thermal controller with a heated lid (MJ Research) under the following conditions: 94°C for 4 min; 35 cycles of 94°C for 1 min; 58°C for 1 min and 72°C for 1 min; and a final PCR extension at 72°C for 7 min. Subsequently, 5 µl of each PCR product were subjected to electrophoresis performed in a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The PCR products in the gel were visualized with UV illumination after staining with ethidium bromide. The amplified products were then purified with the Wizard PCR preps DNA purification system (Promega) according to the manufacturer's protocol. The purified amplicons were subjected to cycle sequencing using the DYEnamic™ ET dye terminator cycle sequencing kit (MegaBACE™, Amersham Biosciences) and the pair of primers mentioned above. The DNA sequences were bidirectionally resolved using a MegaBACE 500 genetic analyzer (Amersham Biosciences) in sequencing mode. The resultant sequences were compared with the ITS sequences in the GenBank database using

BLAST (Basic Local Alignment Search Tool) to generate the best match.

Culture of fungal isolates and chemical extraction of fungal broth. The fungal isolates were inoculated and cultured according to the method described above. After incubation, the cultures were homogenized using a biohomogenizer (m133/1281-0) at 8000 rpm ($125 \times g$) for 2 min. The resultant mixtures were extracted with EtOAc (2×50 ml) and the organic fractions were combined. The solvent was removed using LABOROTA 4002 (HEIDOLPH) at reduced pressure and at 35°C. The residues were weighed and dissolved in EtOAc to a concentration of 25 mg ml^{-1} and then subjected to disc diffusion assay.

Disc diffusion assay. A standard disc diffusion assay (Acar 1980) was used to test the antibacterial properties of the fungal extracts (3 replicates per sample). Samples were prepared by loading 250 μg fungal extract onto each sterile filter paper disc (6 mm in diameter). After the solvent evaporated, the paper disc was placed on the appropriate agar medium, which had already been spread with a suspension of the test bacteria. After incubation for 24 h at 30°C, antibacterial activity was evaluated by measuring the radii of the resultant zones of inhibition from the edge of the filter paper discs. In total, 19 bacterial strains that had originally been isolated from natural marine biofilms in Hong Kong waters were tested. They were B1 (*Vibrio harveyi*), B2 (*Rhodovulum* sp.), B3 (*Pseudoalteromonas* sp.), B4 (*Vibrio hollisae*), B5 (*Vibrio* sp.), B6 (*Shewanella algae*), B7 (*Vibrio fluvialis*), B8 (*Micrococcus luteus*), B9 (*Vibrio furnissii*), B10 (*Staphylococcus haemolyticus*), B11 (*Bacillus licheniformis*), B12 (*Vibrio vulnificus*), B13 (*Staphylococcus aureus*), B14 (*Vibrio halioticoli*), B15 (*Ruegeria* sp.), B16 (*Pseudoalteromonas piscida*), B17 (*Moraxella phenylpyruvica*), B18 (*Vibrio alginolyticus*) and B19 (*Roseobacter* sp.) (Lau et al. 2002, Lee & Qian 2003). To quantify the general antibacterial activity of fungal isolates, a 'total inhibition zone' was calculated by summing up all the inhibition zones of 1 fungal extract against 19 target bacterial strains.

Antifungal assay. The antifungal activity of the 19 target marine bacteria was also tested against fungal isolates (3 replicates per sample). The fungi tested here were first cultivated on a PDA (potato dextrose agar, Sigma) plate at 27°C for 1 wk. A small piece of mycelium was then inoculated in the center of a new PDA plate. The fungi were then cultured at 27°C and allowed to grow to 2 cm in diameter (3 to 5 d). Five μl of the culture of the bacterial strain to be tested were then inoculated around the fungal colony (1 cm to the edge of the fungal colony) and kept at 27°C for 4 to 7 d depending on the growth rate of the fungal strain. For the control, 5 μl of the sterile culture broth without bacterial inocula were used. The experiment was terminated when the fungal colony grew over the control. The distance between the center and the edge of the fungal colony was measured daily. The inhibition of fungal growth was determined by comparing the radii of the fungal colony under the treatment and control conditions.

Statistical analysis. Data were analyzed using the SPSS statistical package. The differences between the treatments and control samples in the antifungal assay were compared using 1-way analyses of variance (ANOVA) followed by Dunnett's test, and the subgroup comparisons were based on Tukey's tests. In all cases, the threshold for significance was 5%.

RESULTS

Fungal isolation and identification

In total, 46 fungal strains were isolated from the seawater samples in this study. These fungal strains were assigned to 17 genera, based on either their morphological characteristics or the ITS sequences (shown in Fig. 1). The dominant genus was *Cladosporium*, followed by *Paraphaeosphaeria*.

Antibacterial activity of fungal extracts

The crude extracts of 32 fungal strains (70% of all tested fungal strains) showed antibacterial activity. The inhibition zones of the active extracts against target bacteria in the primary screening tests are listed in Table 1. The remaining 14 extracts (30% of all extracts) did not show any antibacterial activity (not listed in Table 1). The extracts of 9 fungal strains inhibited only 1 target bacterium. A broad spectrum of antibacterial activity was observed for the extracts of F2, F4, F9, F10, F14 and F35, which inhibited the growth of more than 15 target bacterial strains at a concentration of 250 μg per disc. In contrast, 4 of the target bacteria (B3, B12, B15 and B19) were very sensitive to fungal extracts (Table 1). Among these bacterial species, the *Vibrio vulnificus* Strain B12 was particularly sensitive, as it was inhibited by more than 45% of fungal isolates; B3, B15 and B19 were somewhat sensitive, while B18 was the least sensitive strain, as the growth of this species was inhibited by the extracts of only 4 fungal species.

All *Paraphaeosphaeria* isolates and 79% of *Cladosporium* isolates showed antibacterial activity (Fig. 1). Eight extracts showed strong antibacterial activity with a total inhibition zone of >20 mm and the remaining 24 extracts had weak to moderate antibacterial activity with a total inhibition zone of ≤20 mm
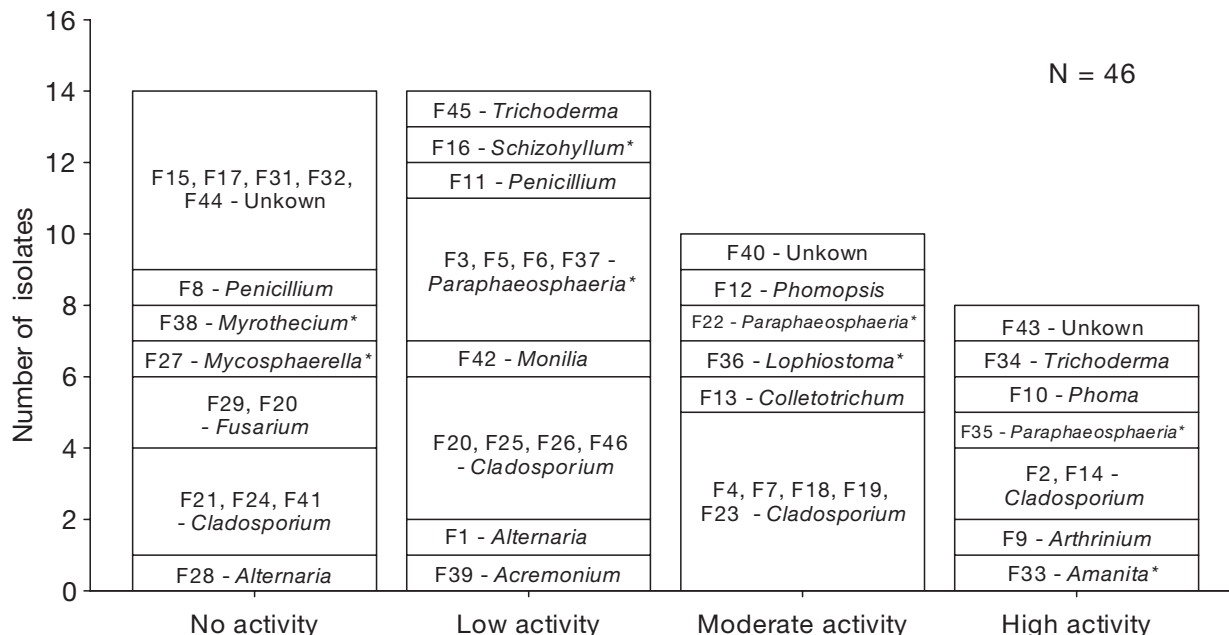
Fig. 1. Generic distribution of the antibacterial activity of fungal isolates. All fungal isolates were identified to the genus level by either morphological examination or a molecular approach. *: isolates identified by the molecular method with the best match from GenBank. Fungal isolates were categorized depending on their antibacterial activity. Low activity: total inhibition zone against 19 target bacteria was <5 mm; Moderate activity: total inhibition zone was between 5 and 20 mm; High activity: total inhibition zone was >20 mm

(Fig. 1). The extracts from Strains F2 (*Cladosporium*), F9 (*Arthrinium*), F10 (*Phoma*), F14 (*Cladosporium*) and F35 (*Paraphaeosphaeria*) not only had strong antibacterial activity, but also inhibited the growth of more than 15 different target bacteria (Fig. 1, Table 1).

Antifungal activity of the target bacterial strains

Eight fungal isolates (17% of all tested fungal isolates) were sensitive to 11 bacterial strains. Among these 8 sensitive fungal isolates, F3, F5, F6, F22, and F35 all belong to the genus *Paraphaeosphaeria* (Table 2). F18 (*Cladosporium* sp.) was the most sensitive fungal strain and its growth was significantly inhibited by 48% of all target bacterial species. The bacterium *Staphylococcus haemolyticus* (B10) showed broader antifungal activity. This bacterium significantly inhibited the growth of all the sensitive fungal strains (Table 2).

The growth rates of all the sensitive fungal strains were similar (the radii of the colonies increased about 3.5 mm d^{-1}), except for 1 fast-growing fungus, F13 (the radii of its colony increased 6 mm d^{-1}). For F13, the inhibition was obvious only 4 d after inoculation; for other sensitive fungal strains, the growth of fungi were inhibited by the bacteria from Day 6, and by Day 8 the inhibition had become very distinct (Fig. 2). Therefore,

the distances on Day 8 were analyzed statistically, except for the fast-growing strain F13, for which the distance on Day 4 was used.

DISCUSSION

Although terrestrial fungi have been the major source of the most important antibiotics in human history for decades, marine fungi have only recently become a focal point of interest for natural product chemists. In this study, many waterborne fungal isolates showed promising antibacterial activity against tested biofilm bacteria, while many of these bacterial strains also exhibited various antifungal activities towards certain fungal strains.

Mangrove ecosystems fringe tropical and subtropical coastlines throughout the world and function as nurseries for a wide variety of marine organisms. It is to be expected that these organisms might furnish many new metabolites (Gloer 1997). In this study, 46 filamentous fungi were isolated from seawater taken from a mangrove stand. They were classified into 2 genera of Ascomycetes, 2 genera of Basidiomycetes, and 12 genera of mitosporic fungi. Due to the difficulties in taxonomic identification of some species of mitosporic fungi (e.g. in the genera *Cladosporium* and *Phoma*), all the species were identified only to the genus level.

Table 1. Inhibition zone (mm) of fungal extracts in a disc diffusion assay (3 replicates per sample). The 19 target bacterial strains were cultivable bacterial colonizers obtained from the South China Sea. B1, B4, B6, B7, B9, B12, B13, B16, B17, B18 were pathogens and the other 9 bacteria actively induced larval settlement of *Hydroides elegans* (Lau et al. 2002, Lee & Qian 2003). Two negative controls were 250 µg of the crude organic extract from blank medium (BM) and 10 µl of the solvent ethyl acetate (EA). The positive control was 25 µg of streptomycin (S)

Fungal extr.	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	Total[a]
F1	–	–	–	0.33	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
F2	1.5	1.17	3.67	3.33	1.42	3.08	0.75	0.58	2.17	–	1.42	5.00	–	2.83	3.17	0.25	1.33	1.17	1.33	17
F3	0.17	–	0.42	–	–	–	–	–	–	–	–	0.58	–	0.25	0.17	–	–	–	–	5
F4	0.17	–	1.17	0.5	–	0.83	0.83	–	1.17	1.33	1.00	2.17	1.08	1.25	1.00	0.67	0.33	2.5	2.67	16
F5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.83	1
F6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–	–	1
F7	–	–	1.17	–	–	0.17	0.83	–	0.08	–	–	1.00	–	0.08	4.33	0.30	–	–	2.33	9
F9	3.33	2.67	7.17	1.33	0.33	2.33	2.67	1.67	2.67	1.17	1.00	4.67	–	4.33	11.83	3.00	3.50	2.17	7.17	18
F10	2.50	0.58	4.17	2.83	1.08	1.75	2.83	0.33	3.92	–	–	7.17	4.50	2.67	1.67	2.75	0.92	–	–	15
F11	–	–	0.33	–	–	–	0.83	–	–	0.25	–	1.50	–	0.50	–	–	–	–	–	5
F12	–	–	1.00	–	–	0.33	0.17	–	0.08	–	–	0.25	–	–	1.17	–	–	–	2.83	7
F13	–	–	0.08	–	–	–	0.92	–	–	–	0.33	–	–	–	2.33	–	–	–	3.50	5
F14	2.67	2.67	4.17	4.17	1.00	1.42	2.67	0.67	3.83	1.50	1.00	5.33	0.50	1.67	3.67	1.08	2.17	–	2.50	18
F16	0.33	–	0.25	0.25	–	–	–	–	0.42	–	–	–	–	–	0.17	–	–	–	–	5
F18	–	–	–	–	–	–	–	–	2.00	3.33	4.50	1.00	–	–	–	–	–	–	–	4
F19	0.17	–	0.25	–	–	0.50	–	0.58	–	–	3.00	1.08	–	–	0.33	–	–	–	5.67	8
F20	0.17	–	0.92	0.42	–	–	–	–	–	–	–	0.42	–	–	–	0.08	–	–	0.67	6
F22	–	–	1.17	–	–	–	–	4.50	–	–	–	0.75	–	–	2.50	–	–	–	–	4
F23	0.17	–	0.67	0.08	–	1.17	–	–	–	0.42	3.17	0.42	–	–	0.17	0.33	–	–	4.83	10
F25	–	–	–	–	–	–	–	–	–	1.67	2.08	0.17	–	–	–	–	–	–	–	3
F26	–	–	–	–	–	–	–	–	1.33	–	–	–	–	–	–	–	–	–	–	1
F33	1.50	1.67	1.50	–	1.67	1.50	–	–	0.50	–	–	2.33	0.67	–	4.67	2.67	1.00	0.83	5.17	13
F34	2.33	–	1.67	–	–	–	–	4.83	4.17	4.83	5.17	0.25	3.17	–	4.67	–	–	–	–	9
F35	0.08	1.67	0.50	1.83	0.33	0.08	–	0.67	–	0.50	1.67	0.83	2.00	1.17	4.33	1.83	–	–	2.83	15
F36	–	–	–	2.00	–	–	–	1.33	–	1.75	1.67	–	1.33	–	–	–	–	–	0.42	6
F37	–	–	–	–	–	–	–	–	1.83	–	–	–	–	–	–	–	–	–	–	1
F39	–	–	–	0.42	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
F40	–	–	–	–	–	–	–	–	1.00	0.83	3.17	0.08	–	–	–	–	–	–	2.17	5
F42	–	–	–	–	–	–	–	–	–	–	–	0.17	–	–	–	–	–	–	–	1
F43	–	–	1.00	2.33	–	0.67	0.67	2.50	3.00	2.83	2.00	2.17	2.83	–	0.67	0.50	–	–	1.42	13
F45	–	–	–	0.75	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
F46	3.17	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1
BM	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
EA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0
S	2.08	2.50	2.50	1.00	2.67	3.33	1.00	3.67	2.17	2.67	1.17	2.33	1.33	3.33	5.33	3.17	4.17	2.67	3.67	19
Total[b]	14	6	19	14	6	12	10	10	15	12	14	21	8	9	18	11	6	4	16	

[a]Number of target bacteria inhibited by a particular fungal extract
[b]Number of fungal extracts showing the inhibitive effect to a particular target bacterium

In this study, the number of strains identified as Ascomycetes was much lower than that of mitosporic fungi, which deviates from previous predictions (Kohlmeyer & Volkmann-Kohlmeyer 1991). It is highly possible that most of the fungal isolates obtained in this study were not obligate but facultative marine fungi, or even terrestrial fungi that were able to grow under the isolation conditions applied here.

A special bioassay system for screening the antibacterial activity of fungal isolates was established in this study. Nine species of pathogens and 10 species of ecologically relevant bacteria (effectively inducing larval settlement of *Hydroides elegans*) obtained from natural marine biofilm were used as target bacteria to screen for antibacterial activity in extracts of marine fungi. Compared to the minimum inhibitory concentration (MIC) assay (Torres et al. 2002) and agar diffusion assay (Kubanek et al. 2003), the disc diffusion assay used here has some shortcomings. For example, the size of the inhibition zone might be affected by the ability of active compounds on the paper disc to diffuse through the agar, thus leading to a possible underestimation of antimicrobial activity. Furthermore, small inhibition zones could also be due to the artefacts such as physical effects of the extracts and might not be due to the presence of antibiotics. However, this assay is still a simple, fast and effective method and has been widely used for antibiotic activity screening. About

Table 2. Inhibitive effects of bacterial species on fungal growth on Day 8 (for fast-growing fungus F13, the inhibitive effect on Day 4 was analyzed). Control (C) was blank culture medium. Activities of bacterial species were assessed by 1-way ANOVA (**p < 0.001) and post hoc comparison tests. Tukey's tests were used to determine if there were any significant differences among treatment groups; groups that were not different at the 0.05 level were joined by an underline. All the groups were arranged in descending rank order from left to right, based on their mean values. Dunnett's tests were used to detect significant difference (marked by *) between each of the treatments and the control

Fungus	C	B2	B4	B7	B8	B10	B11	B12	B13	B16	B17	B19	ANOVA $F_{11,24}$	Dunnett's and Tukey's results
					Radii of fungal colonies (mm)									
F3	26.3	26.0	26.8	19.3	22.3	20.3	20.9	22.5	20.1	26.0	26.3	26.7	41.2**	B7 B13* B10* B11* B8* B12* B16 B2 B19
F5	25.5	25.7	26.0	18.4	23.3	21.0	21.7	22.3	20.5	25.7	25.2	25.7	40.9**	B7 B13* B10* B11* B12* B8* B17 B16 B2 B4
F6	18.3	17.6	18.2	15.8	18.0	16.1	16.1	16.7	16.7	16.0	15.7	15.8	4.62**	B7* B16* B11* B10* B12 B13 B17 B2 B19 B8 B4
F13	26.8	27.3	27.3	26.8	27.2	23.4	24.0	24.2	25.8	25.4	26.8	27.2	14.2**	B10* B11* B12* B13 B16 B7 B17 B8 B19 B4 B2
F18	20.3	17.6	20.2	15.7	17.8	18.1	16.7	15.9	16.1	15.7	20.5	17.7	28.7**	B7* B16* B12* B13* B11* B2* B19* B8* B10* B4 B17
F22	18.7	19.0	15.7	19.0	19.5	16.5	17.0	15.7	16.5	15.9	19.0	19.0	14.2**	B12* B4* B16* B10* B11 B7 B17 B19 B2 B8
F27	19.5	19.0	13.5	16.1	17.9	14.3	14.4	14.3	14.6	14.	18.5	19.0	36.4**	B4* B16* B10* B12* B11* B13* B7* B8 B17 B19 B2
F35	20.0	20.0	19.0	16.5	19.8	17.3	19.3	20.3	17.5	20.5	20.0	15.5	17.3**	B19* B7* B10* B13* B11* B8 B11 B8 B17 B2 B12 B16

70% of the fungal species tested, which belonged to 13 different genera, produced antibacterial compounds. In comparison to other antibacterial activity screening against the standard pathogens *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* (Cuomo et al. 1995, Christophersen et al. 1999), the percentage reported for marine fungi is much higher. One possible explanation is that fouling bacteria might be more sensitive to fungal extracts than standard pathogens are. However, the higher percentage could also be due to the larger number of target organisms as well as the high concentration of extracts used in this study. The power of bioassay screening is highly dependent on the concentration of the testing extracts and the number of target species used in the bioassay. Studies based on very small number of target species and low testing concentration may lead to biased conclusions and should be avoided in primary screening.

In this study, more than 30% of the isolates belonged to the genus *Cladosporium* and the extracts of 79% of the *Cladosporium* strains showed antimicrobial activity, which was also very high. Since this genus consists of over 200 species, a large array of secondary metabolites is likely to be produced by this group of fungi. However, there was no correlation between the sensitivity of bacterial isolates to fungal extracts and bacterial phylogenetic grouping. Several target *Vibrio* strains showed different sensitivity to the same fungal extracts. For example, bacterial Strain B12 (*V. vulnificus*) was the most sensitive strain and could be inhibited by the extracts of more than 45% of the fungal species, while another *Vibrio* strain (*V. alginolyticum*) was sensitive only to the extracts of 4 fungal species.

Since many fungal isolates did not sporulate in cultures, it was difficult to evenly develop fungal film on agar plates or to prepare a spore suspension and then to observe the spore germination. Therefore, common antifungal assays, such as the MIC assay (Koh et al. 2002) and standard disc diffusion assay (Petitt et al. 1998), were not really suitable for this purpose. Following closely the method described by Holmstroem et al. (2002), a modified assay was established in this study. This method was much easier to manipulate, and contamination could be better avoided. However, the inhibition of fungal growth on agar plates may also be due to the nutrient competition between bacteria and fungi because live bacteria were used in this bioassay. Results of the antifungal assay showed that only 8 fungal isolates (17% of all tested fungi) were significantly inhibited by some target bacterial strains. If nutrient competition affects the result that much, the percentage of sensitive fungal isolates should be much higher. In addition, apart from active bacterial species, when test fungal isolates encounter other bacteria on agar
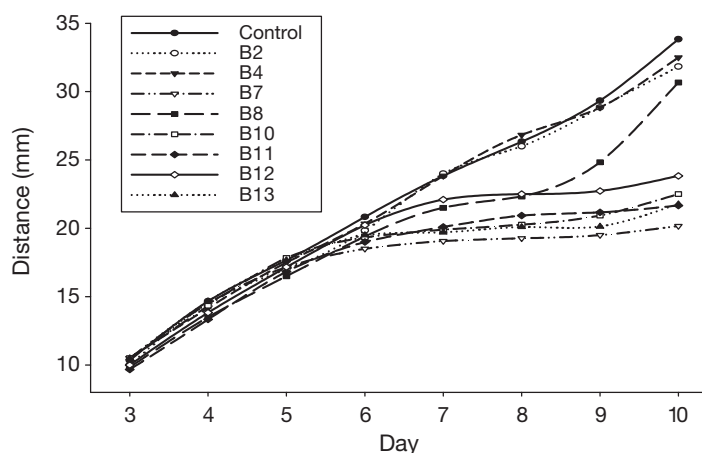
Fig. 2. Antifungal activities of active target bacterial strains against fungal isolate F3 *Paraphaeosphaeria* sp. Different curves show the growth of F3 under the suppression of target bacterial strains. The control was blank medium

plates, their mycelia just extend over the bacterial colonies without any hindrance. Therefore, the inhibition of fungal growth should be mainly due to the inhibitive effect of active bacterial species rather than the nutrient competition.

Among the 8 sensitive fungal isolates, 5 strains belonged to the same genus *Paraphaeosphaeria.* Two *Staphylococcus* bacteria (B10 and B13) showed broad antifungal activity, significantly inhibiting the growth of more than 6 sensitive fungal strains. There seems to be a correlation between the antifungal activity or sensitivity of test microbes and their phylogenetic grouping.

In this study, we found some unexpected results. Certain fungal isolates inhibited the growth of particular target bacterial strains, while these fungal isolates could also be inhibited by the same bacterial strains. For instance, fungal isolates F18, F22 and F35 showed high antibacterial activity against bacterial strains B10 and B11, B8, B13 and B19, respectively, while they were also significantly inhibited by these bacterial strains (Tables 1 & 2). This result might be due to the different culture method used for fungal growth in the 2 assays: in the disc diffusion assay, we screened antibacterial activity of fungal extracts that were obtained from liquid fungal cultures, while in the antifungal assay tested fungal isolates were cultured on agar plates. Sensitive antibacterial fungi may produce active antibacterial compounds when cultured in a liquid medium; however, the quantity of the active compounds may decrease dramatically when the fungal strains are cultured on agar plates. It was also found that although some bacteria species inhibited the growth of certain fungal isolates, the extracts from liquid culture of most of these active bacteria did not

show antifungal activity (data not shown), suggesting that these bacteria may only produce active antifungal compounds when cultured on agar plates. Similar results have been documented with other strains in our laboratory, e.g. a bacterial strain, *Pseudoalteromonas peptidysin*, produced active metabolites when cultured on an agar plate, but the activity of the bacterial extracts was basically lost when the bacterium was cultured in a liquid medium (Yang et al. unpubl. data).

The fungal strains tested in this study were isolated from seawater in mangrove stands. Recent work in our laboratory showed that most of these fungal strains could also be isolated from natural biofilm, such as many species of *Arthrinium*, *Cladosporium*, *Paraphaeosphaeria* and *Phoma* (authors' unpubl. data). Therefore, from the results of antimicrobial activity screening, it was found that antagonistic antimicrobial activity widely exists between common fouling bacteria and fungi, suggesting that the competitive interaction between fungi and bacteria during the initial development of biofilms may also affect the chemical signals produced by the film and cannot be neglected when studying the development and function of biofilms.

Based on these results, we concluded that (1) a high percentage of fungal isolates from seawater around mangroves showed antibacterial activity against microfouling bacteria, suggesting that marine fungi can be a source of natural antimicrofouling compounds; and (2) many common fouling bacteria also showed inhibitive effects against certain marine fungi, suggesting a competitive interaction between marine fungi and bacteria during the initial development of natural biofilms.

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