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The role of epibotic bacteria from the surface of the soft coral *Dendronephthya* sp. in the inhibition of larval settlement

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Abstract

It has been suggested that bacteria associated with soft-bodied organisms are suggested to produce bioactive compounds against the attachment of invertebrate larvae and bacteria onto the surface of these organisms. Our recent study has demonstrated that epibiotic bacteria from the surface of the soft coral *Dendronephthya* sp. (Coelenterata: Octocoralia, Alcyonacea) inhibit the growth of bacteria commonly found in marine natural biofilms. In the present study, the effect of 11 epibiotic bacteria isolated from the surface of *Dendronephthya* sp. on larval settlement of the tubeworms *Hydroides elegans* was examined using laboratory bioassay. Among 11 bacterial isolates, 2 strains (18%) inhibited the larval settlement of *H. elegans* (Haswell), 4 strains (36%) were ''inductive'' to larvae and the remaining 5 strains (46%) were ''non-inductive''. There was no correlation between the antifouling activities of bacterial isolates and their phylogenetic origin, i.e. closely related bacterial strains showed different effects on larval settlement of *H. elegans*. When all ''inductive'', ''non-inductive'' and ''inhibitive'' bacterial isolates were mixed in a 1:1:1 ratio, the effect of the resultant multispecies film on larval settlement became ''inhibitive''. Waterborne compounds of *Vibrio* sp. and an unidentified α-*Proteobacterium*, which suppressed the settlement of *H. elegans* and *Bugula neritina* (L.) larvae, were further investigated using size fractionation and bioassay-guided enzymatic analysis. It was found that antilarval settlement compounds from these bacteria were heat-stable polysaccharides with a molecular weight >100 kDa. The results indicate that the bacteria associated with the soft coral *Dendronephthya* sp. may contribute to the antifouling mechanisms of the soft-bodied organisms

Abbreviations: CSW, conditioned seawater; AFSW, autoclaved filtered seawater; NBF, natural biofilm; DAPI, DNA-binding fluorochrome 4,6-diamidino-2-phenylindole; IBMX, 3-isobutyl-1methylxanthine.

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by producing compounds that are against bacterial growth and settlement of macrofoulers on the surface of their host.

Keywords: Antifouling; *Bugula neritina*; Carbohydrates; *Dendronephthya* sp.; Epibiotic bacteria; Larval settlement; *Hydroides elegans*; Soft coral

1. Introduction

Microorganisms, larvae of fouling invertebrates and algal spores rapidly colonise animate and inanimate marine surfaces in tropical waters. Biofouling on the surface of living organisms (called epibiosis) causes damage, inhibition of growth, necrosis and even death of basibionts (Mitchell and Chet, 1975; Sand-Jensen, 1977; Dixon et al., 1981; Wahl, 1997). Soft-bodied marine organisms such as soft corals, algae, sponges and ascidians developed effective protection against macrofouling (Wahl et al., 1994; Slattery et al., 1995; Kelman et al., 1998; Harder and Qian, 2000; Hentschel et al., 2001; Dobretsov and Qian, 2002; Harder et al., 2003).

Marine biofilms developed in natural environment, which are often composed of many species of marine bacteria, diatoms, algal spores and macromolecules and developed in the laboratory with single pure bacterial culture can enhance (Kirchman et al., 1982, Mitchell and Maki, 1988; Lau and Qian, 1997) or inhibit larval settlement (Maki et al., 1988; Holmström et al., 1992; Egan et al., 2000, 2001; Dobretsov and Qian, 2002; Lau et al., 2003). We hypothesized that soft-bodied organisms may control biofouling on their surfaces by regulating the bacterial species composition of the biofilm through suppressing inductive bacterial strains and enhancing the growth of non-inductive ones. The production of a range of inhibitive compounds by the bacterium *Pseudoalteromonas tunicata*, isolated from the surface of the tunicate *Ciona intestinalis* (Holmström et al., 1992; James et al., 1996) appears to support this argument. The compounds reduced growth of numerous marine bacteria and settlement of larvae of the barnacle *Balanus amphitrite*, the polychaete *H. elegans*, fungi, as well as algal spores of the *Ulva lactuca* and *Polysiphonia* sp. (Holmström and Kjelleberg, 1999; Egan et al., 2000, 2001; Holmström et al., 2002). In a recent study, we demonstrated that bacteria *Vibrio* sp. and *Pseudoalteromonas* sp. isolated from the surface of the seaweed *Ulva reticulata* inhibited the growth of the benthic diatom *Nitzschia paleacea* and the settlement of *H. elegans* larvae (Dobretsov and Qian, 2002).

The ability of soft corals (Coelenterata: Octocoralia, Alcyonacea) to control micro- and macrofouling has been attributed to the excretion of slime and production of secondary metabolites (Slattery et al., 1995; 1997; Aceret et al., 1998; Kelman et al., 1998; Wilsanand et al., 2001). The soft coral *Dendronephthya* sp. is one of the species from the South China Sea that remains free from biofouling all year. Alcohol extracts of *Dendronephthya* sp. inhibited the settlement of *B. amphitrite* cyprids and byssus production of the bivalve *Perna viridis* (Kawamata et al., 1994; Wilsanand et al., 1999). Furthermore, the active compound was identified as trigonelline of the betaine group.

In our previous study, we isolated 11 bacterial strains from the surface of the soft coral *Dendronephthya* sp. and tested their antibacterial effect (Harder et al., 2003). These strains

Table 1
Phylogenic status of bacterial isolates from the soft coral *Dendronephthya* sp.

Abbreviation	Strain designation	Closest match in Genbank (GenBank accession no)	Similarity (%)
γ-Proteobacteria			
C1	UST991130-041	*Pseudoalteromonas* sp. (AJ391189)	97
C2	UST991130-040	*Vibrio* sp. (AJ391203)	99
C3	UST991130-048	*Vibrio* sp. (AJ391203)	98
C4	UST991130-046	*Vibrio ichthyoenteri* (AJ4211445)	97
C5	UST991130-038	Unidentified *γ-Proteobacterium* (AB010855)	98
C6	UST991130-039	Unidentified *γ-Proteobacterium*(AB010844)	99
α-Proteobacteria			
C7	UST991130-037	Unidentified *α-Proteobacterium* (AB026194)	99
C8	UST991130-043	Unidentified *α-Proteobacterium* (AB026194)	98
C9	UST991130-042	Uncultured *Ruegeria* (AF259606)	98
Cytophaga–Flexibacter–Bacteroides			
C10	UST991130-045	*Flexibacter* sp. (AB058905)	96
C11	UST991130-047	*T. mesophilum* (AB032504)	98

belong to three major groups of bacteria: α- and γ-Proteobacteria and *Cytophaga–Flexibacter–Bacterioides* (Table 1). Organic extracts of *Dendronephthya* sp. and waterborne products of coral-associated bacteria distinctly inhibited the growth and attachment of benthic bacteria. However, the antilarval settlement activity of those bacteria has not been investigated. The objectives of the current study were: to investigate the effect of mono- and multispecies bacterial films on larval settlement of *Hydroides elegans*; to study the effect of waterborne compounds excreted by coral-associated bacteria on the settlement of *H. elegans* and *Bugula neritina* larvae; and to investigate the chemical nature of compounds of inhibitive bacteria against larval settlement.

2. Materials and methods

2.1. Larval culture

Adult *H. elegans* tubeworms and *B. neritina* bryozoan were collected from submerged rafts at the fish farms in Long Harbour and Yung Shue O, Hong Kong (114°21′ E, 22°24′ N). Larval cultures were prepared and maintained accordingly to Bryan et al. (1997). Newly hatched bryozoan larvae and competent tubeworm larvae were used in the bioassays. Competence of the *H. elegans* larvae was determined by their morphology and by a bioassay with 3-isobutyl-methylxanthine (IBMX) at 10^{-4} M solution in autoclaved filtered (0.22 μm) seawater (AFSW) (Pechenik and Qian, 1998).

2.2. Preparation of bacterial films and bacterial-conditioned seawater (CSW)

Before the onset of the experiments, 11 bacterial strains (Table 1) previously isolated from soft coral (for details, see Harder et al., 2003) were grown to the stationary phase in

tubes with 0.5% bacteriological peptone (Oxoid) and 0.3% yeast extract (Oxoid) in AFSW at 30 ± 1 °C, under a cycle of 15-h light/9-h darkness. Bacteria in culture suspension were harvested by centrifugation (6000 × *g* for 15 min). Cell pellets were washed with AFSW and used for the development of bacterial films.

To prepare the bacterial films, the cell pellets were washed and resuspended in AFSW to an optical density of 0.8 (Abs) at a wavelength of 610 nm (Shimatzu, China). Five milliliters of the bacterial suspension containing either 1 or 11 species of bacteria at equal volume ratios was introduced into polystyrene dishes and incubated for 3 h at room temperature to allow the attachment of bacterial cells onto the dish surface. After the incubation, unattached bacteria were decanted and the dishes were rinsed several times with AFSW and used for bioassays or for the preparation of waterborne bacterial compounds (hereafter called conditioned seawater, CSW).

To obtain CSW from bacterial strains C2 and C8, 5 ml of AFSW was added to each polystyrene dish covered by bacterial films. Dishes were incubated for 12 h at 23 °C. Thereafter, the CSW was sterile-filtered (Millopore, 0.22 μm) and used for the experiments.

2.3. Laboratory assays of larval settlement

The laboratory bioassay was performed several times on different larval batches with replication ($n = 5$) in sterile polystyrene dishes (#430588, Corning, USA) containing 20 larvae of *H. elegans* or *B. neritina* and the sample under investigation or the biofilm. The experiments with enzyme-treated CSW (see below) were conducted with replication ($n = 6$) in multiwell polystyrene dishes (#3047, Falcon, USA) containing 10 larvae of *B. neritina*. In all bioassays, AFSW were used as the negative control whereas 6-day-old natural biofilm (NBF) was used as a positive additional control in the experiments with *H. elegans*. In all bioassays with CSW from bacteria and the larvae of *H. elegans*, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, Fluka) at 10^{-4} M in AFSW was used as an artificial stimulator for the larval settlement (Lau and Qian, 1997; Pechenik and Qian, 1998). Before the experiments, *H. elegans* larvae were treated with IBMX for 1 h, then washed in AFSW and used in bioassays with CSW from bacteria. Larval settlement assays were run at 28 °C under continuous illumination for 24 h in the case of *H. elegans* and for 1 h without illumination in the case of *B. neritina*. After the testing periods, the dishes were emptied and the settled juveniles were counted with a microscope. In some treatments, the bacterial abundance in the experimental dishes was determined after the bioassays. The attached bacteria were visualised with the DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Fluka Chemie, Switzerland) at 0.5 μg ml^{-1}. Formalin-fixed (4% in AFSW) dishes ($n = 2$) were rinsed with AFSW and stained with DAPI for 15 min. The number of bacteria in five randomly selected fields of view was then counted under an epifluorescence microscope (Axiophot, Zeiss, Germany; magnification 1250×; $\lambda_{Ex} = 359$ nm, $\lambda_{Em} = 441$ nm).

2.4. Separation of CSW sample

To purify and estimate the molecular weight range of inhibitory compounds from the CSW obtained from bacteria C2 and C8, a series of ultrafiltration steps through

YM-100, YM-10 and YM-1 molecular weight cutoff membranes (Millipore, USA) were performed in decreasing order. This procedure yielded filter residues of 100, 10 and 1 kDa molecular weight cutoff and a 1 kDa filtrate. Retentates were redissolved in AFSW to the original volume of CSW. The filtrates and residues were collected, filtered (0.22 μm, Millipore), and thereafter assayed for the ability to inhibit the settlement of *H. elegans* larvae. In retentates, greater than 100 kDa protein and carbohydrate contents were determined spectrophotometrically according to Bradford (1976) and Dubois et al. (1956) with standards of bovine serum albumin and glucose, respectively.

2.5. Enzyme and heat treatments of CSW

A variety of enzyme probes was used in the digestion experiments of the greater than 100 kDa fraction of CSW from bacteria C2 and C8 to reveal the structural components of the biological activity. The concentrated stock solutions of the following enzymes were prepared in 10 mM Tris–HCl (pH 7) in 50% glycerol and diluted with CSW (enzyme treatment) or AFSW (enzyme blank). The following working concentrations were then prepared from the stock solutions: 0.01 mg ml^{-1} trypsin (from bovine pancreas), 0.2 mg ml^{-1} papain (from *Papaya latex*), 0.5 mg ml^{-1} sulfatase (from *Patella vulgata*), and 0.1 mg ml^{-1} α-amylase (from *Aspergillus orysae*). All enzymes were purchased from Sigma, USA. The enzymes and the CSW were incubated at 37 °C for 30 min and the samples were used without further purification in larval settlement assays with *B. neritina*. In addition, CSW from bacteria C2 and C8 was heated at 95 °C for 15 min and used in the larval settlement bioassay with *B. neritina* and *H. elegans*. For the control, heated AFSW was used.

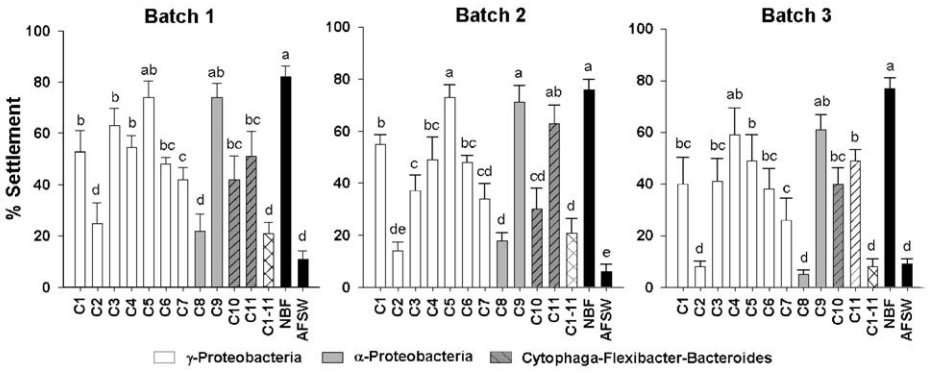

Fig. 1. Larval settlement of *H. elegans* in response to the bacterial isolates from the soft coral *Dendronephthya* sp. (after 24 h). The bioassay was performed in triplicate (denoted as Batches 1, 2 and 3). Autoclaved filtered seawater (AFSW) and natural biofilm (NBF) were used as negative and positive controls, respectively. The monospecies bacterial films are C1, C2, C3, C4, C5, C6, C7, C8, C9, C10 and C11. The multispecies bacterial film is indicated by abbreviation C1–11. Data plotted are means ± S.E. of five replicates. Data that are significantly different in LSD test ($P < 0.05$, one-way ANOVA) are indicated by different letters above the bars.

2.6. Statistical analysis

The numbers of settled larvae were converted into percentages that were then arcsine-transformed. In the case of zero metamorphosed larvae, a value of $1/4n$ (n = number of larvae in a single replicate) was assigned to improve the arcsine transformation (Zar, 1996). The normality assumption was verified with the Shapiro–Wilk's test (Shapiro and Wilk, 1965). The differences between the treatments and the control were determined by one-way ANOVA followed by LSD post hoc test (Zar, 1996). When the data failed the normality assumption, they were analysed nonparametrically. This was achieved by transformation of values to ranks and followed by the Kruskal–Wallis test and later by an advanced multicomparison U-statistic (STP, Sokal and Rohlf, 1981). The density of the bacteria was square-root-transformed in order to ensure normality of variance. It was analysed with one-way ANOVA followed by the LSD post hoc test. In all cases, the threshold for significance was 5%. The data presented in all the figures herewith are not transformed.

3. Results

3.1. Laboratory assays of larval settlement

3.1.1. Effect of monospecies and multispecies bacterial films on settlement of H. elegans

In the presence of the bacterial strains isolated from the soft coral, the settlement of *H. elegans* larvae were significantly different (Fig. 1, batch 1 ANOVA: $F_{13,56} = 11.311$, $P < 0.0001$; batch 2 ANOVA: $F_{13,56} = 17.343$, $P < 0.0001$; batch 3 ANOVA: $F_{13,56} = 12.099$, $P < 0.0001$). LSD test results classified all tested bacterial strains into the following three groups: "inductive" bacteria that invoked larval settlement at the same

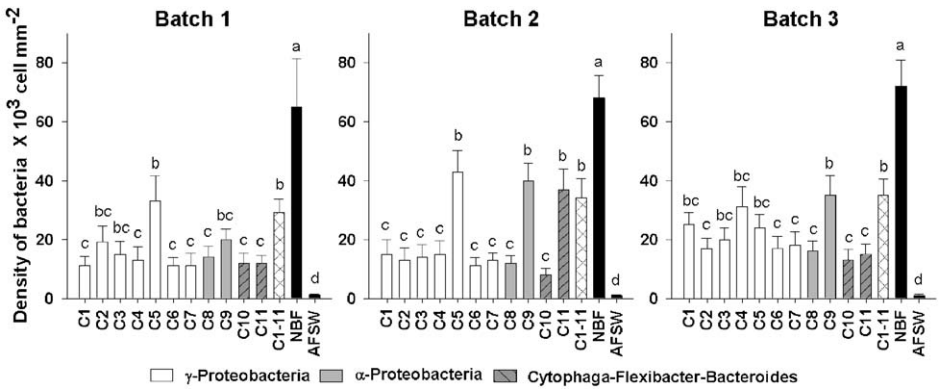

Fig. 2. Mean number of attached bacteria (cell mm^{-2}) in experiments with attachment of *H. elegans* larvae. The larval bioassay was performed in triplicate (denoted as Batches 1, 2 and 3). The monospecies bacterial films are C1, C2, C3, C4, C5, C6, C7, C8, C9, C10 and C11. Tested multispecies bacterial film is indicated by abbreviation C1–11. Data plotted are means ± S.E. of five replicates. Data that are significantly different in the LSD test ($P < 0.05$, one-way ANOVA) are indicated by different letters above the bars.

level ($P>0.05$) as in NBF (positive control), ''inhibitive'' bacteria that led to the same larval settlement as in unfilmed dishes (negative control), and ''non-inductive'' bacteria that did not fall into either one of these two categories. Under these arbitrary criteria, bacterial films of C9 (batches 1, 2 and 3), C5 (batches 1 and 2), C11 (batch 2) and C4

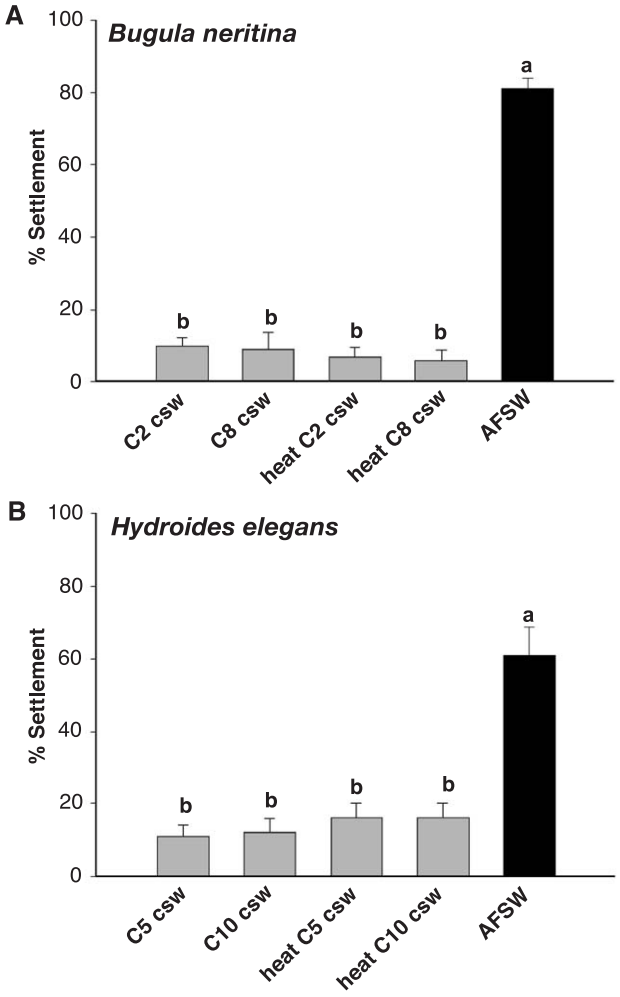

Fig. 3. (A) The effect of conditioned seawater and heat-treated (95 °C for 15 min) conditioned seawater from the bacteria *Vibrio* sp. (C2 CSW) and the unidentified α-*Proteobacterium* (C8 CSW) on larval attachment of *B. neritina* (after 1 h) in comparison to a control (autoclaved filtered seawater (AFSW)). (B) Larval attachment of *H. elegans* (after 24 h) in response to conditioned seawater and heat-treated (95 °C for 15 min) conditioned seawater from the bacteria *Vibrio* sp. (C2 CSW) and the unidentified α-*Proteobacterium* (C8 CSW). Before this experiment, the larvae were treated with IBMX at 10^{-4} M for 1 h, then washed in AFSW and used in the bioassay. AFSW was used as a control. Data plotted are means ± S.E. of five replicates. Data that are significantly different in the LSD test ($P<0.05$, one-way ANOVA) are indicated by different letters above the bars.

(batch 3) were "inductive", while films of C2 (batches 1–3) and C8 (batches 1 and 3) were "inhibitive". Other bacterial strains (C1, C3, C6, C7 and C10) showed a moderate settlement rate. The "inhibitive" bacterial strains (C2 and C8) belong to γ- and α-Proteobacteria phylogenetic branches (Table 1; Fig. 1) while the "inductive" bacterial strains were affiliated to the γ- and α-Proteobacteria and *Cytophaga–Flexibacter–Bacteroides* phylogenetic branches. "Non-inductive bacteria" were found in all taxonomic groups.

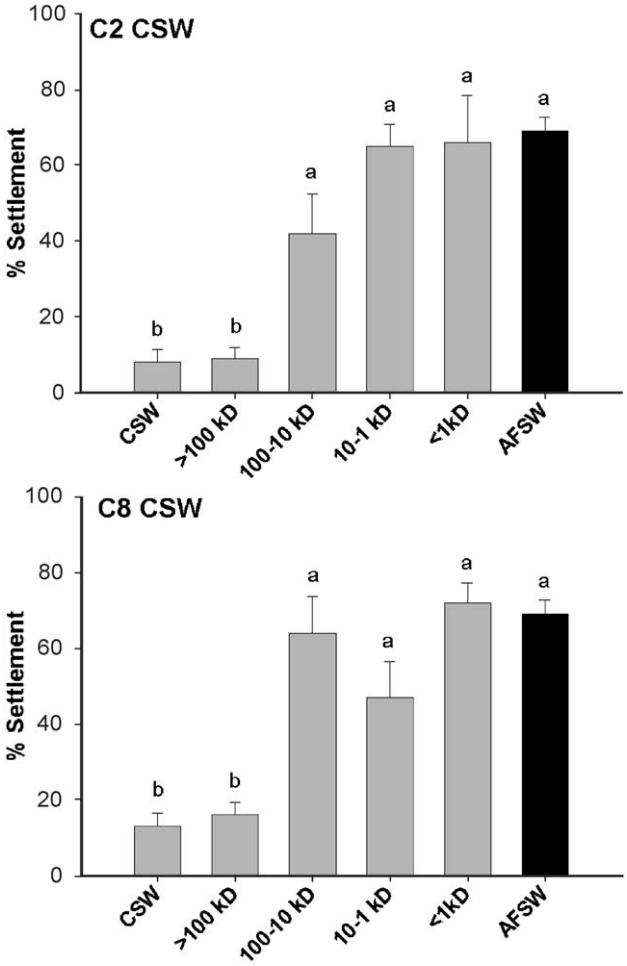

Fig. 4. The effect of molecular cutoff fractions of conditioned seawater from the bacteria *Vibrio* sp. (C2 CSW) and the unidentified α-*Proteobacterium* (C8 CSW) on larval attachment of *H. elegans* (after 24 h). Before the experiment, the larvae were treated with IBMX at 10^{-4} M for 1 h, then washed in AFSW and used in the bioassay. AFSW was used as a control. Data plotted are means ± S.E. of five replicates. Data that are significantly different in Kruskal–Wallis STP test ($P<0.05$) are indicated by different letters above the bars.

The effect of the alteration of bacterial community composition on larval settlement was investigated by mixing bacterial isolates for bacterial film development. When 11 bacterial isolates were mixed at equal ratios, the low level of the larval settlement of *H. elegans* was recorded (Fig. 1). Attachment rate on the multispecies film was of the same as that for AFSW and for the monospecies bacterial film of C2 and C8 (LSD, $P>0.05$).

Epifluorescence microscopy revealed that the density of bacteria on the experimental dishes were significantly different (Fig. 2, batch 1 ANOVA: $F_{13,126}=4.014$, $P<0.0001$; batch 2 ANOVA: $F_{13,126}=9.623$, $P<0.0001$; batch 3 ANOVA: $F_{13,126}=6.064$, $P<0.0001$). The highest density of bacteria was found on the dishes filmed with NBF while the second highest density of bacteria was found on the dishes with monospecies films of C1, C2, C4, C5, C9 and C11, as well as with the multispecies bacterial film. Other bacterial films had moderate densities of bacteria. The lowest density was observed in the dishes with AFSW.

3.1.2. Effect of CSW and heated CSW from the C2 and C8 strains on settlement of H. elegans and B. neritina

Additional settlement bioassays were conducted on the "inhibitive" bacterial films (i.e. C2 and C8) in order to investigate the chemical nature of the antifouling compound. CSW from bacteria C2 and C8 significantly inhibited settlement of *B. neritina* larvae (Fig. 3A; ANOVA: $F_{4,20}=31.40$, $P<0.0001$; LSD test: $P<0.001$). The effect of heated CSW was not significantly different (LSD test: $P=0.4$) from that of untreated CSW.

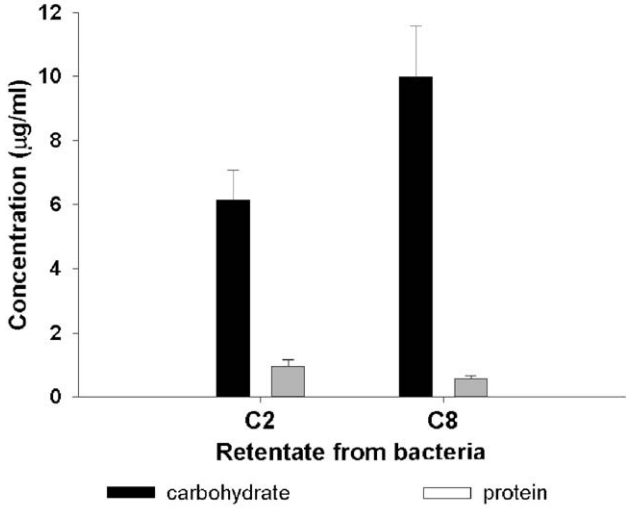

Fig. 5. Protein and carbohydrate concentrations in the greater than 100 kDa retentates of CSW obtained from bacteria. Data plotted are means ± S.E. of five replicates.

The one-way ANOVA indicated that the settlement of *H. elegans* larvae induced by IBMX was inhibited by CSW from the C2 and C8 strains (Fig. 3B; ANOVA: $F_{5,24} = 15.642$, $P < 0.0001$). Heating of CSW had no significant (ANOVA and LSD test: $P < 0.001$) effect on the activity of the compound. The highest larval settlement rate was observed in AFSW.

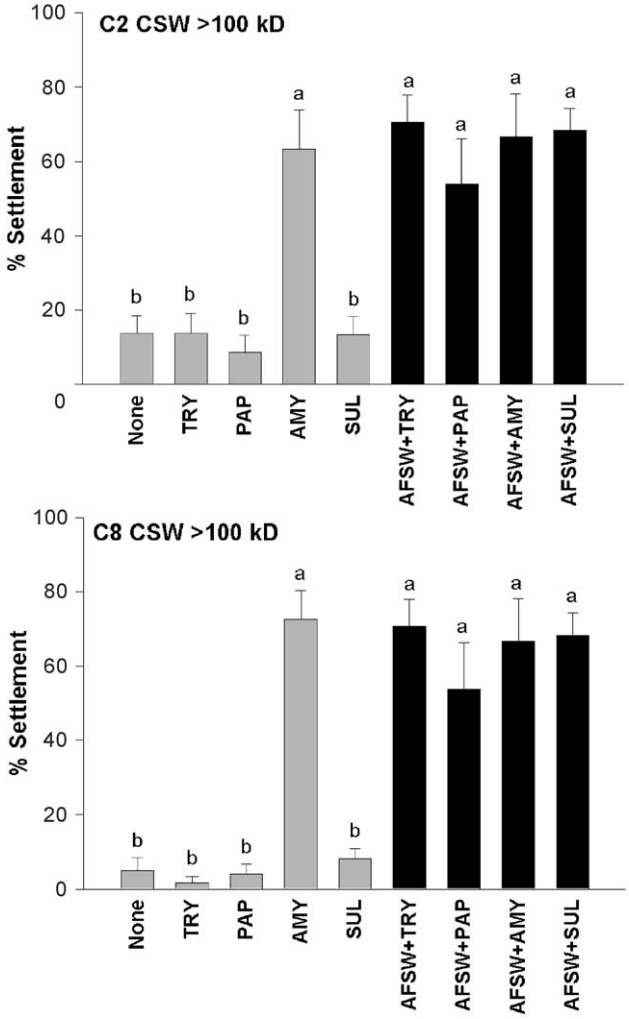

Fig. 6. The effect of enzyme treatment of the greater than 100 kDa fraction of conditioned seawater from bacteria *Vibrio* sp. (C2 CSW >100 kDa) and the unidentified *α-Proteobacterium* (C8 CSW >100 kDa) on larval attachment of *B. neritina* (after 1 h) in comparison to a positive control (AFSW) and a negative control (None). Enzymes used were trypsin (TRY), papain (PAP), sulfatase (SUL) and α-amylase (AMY). Data plotted are means ± S.E. of five replicates. Data that are significantly different in the LSD test ($P < 0.05$, one-way ANOVA) are indicated by different letters above the bars.

3.2. Size fractionation of CSW from the C2 and C8 strains

The larval settlement of *H. elegans* induced by IBMX in the presence of molecular fractions of CSW from bacteria was different (Fig. 4; Kruskal–Wallis: $P < 0.0001$). The larval settlement induced by IBMX in the dishes containing the fractions 100–10, 10–1 and less than 1 kDa of CSW from C2 was the same as in the AFSW (STP: $P < 0.05$), while the settlement in the dishes containing the 100 kDa filter residues was significantly lower than in the other dishes (STP: $P < 0.01$). Analogous results were obtained for the CSW from the C8 strain. In both cases, the 100 kDa residue inhibited larval settlement, while other fractions had no effect on larval settlement (Fig. 4).

Protein and carbohydrate contents in the 100 kDa residue showed that this fraction mainly consisted of carbohydrates (Fig. 5). The concentration of proteins was higher in the cutoff of the 100 kDa residue obtained from C2 compared to the retentate from C8.

3.3. Enzyme treatments of CSW from the C2 and C8 strains

After the incubation of CSW from the C2 and C8 strains with different enzyme probes, the larval settlement of *B. neritina* differed significantly among the treatments (Fig. 6; ANOVA: $F_{8,45} = 13.067$, $P < 0.0001$ for C2 and $F_{8,45} = 17.679$, $P < 0.0001$ for C8). Enzyme blanks had no effect on larval settlement. In CSW from bacteria C2 and C8, the percentage of larval settlement in the treatments with amylase was significantly higher than that in the CSW treatment (LSD test, $P < 0.001$) but statistically the same as in the AFSW control (LSD test: $P > 0.05$). On the contrary, the percentage of larval settlement in the treatments with trypsin, papain and sulfatase was the same as that in the CSW control (LSD test: $P > 0.05$).

4. Discussion

It has been demonstrated in previous studies that the soft corals *Dendronephthya* sp. can control macrofouling on its surface by the production of secondary metabolites (Kawamata et al., 1994; Wilsanand et al., 1999). Water-soluble, low-molecular-weight substances from the soft coral *Dendronephthya* sp. inhibited the settlement of larvae of *B. amphitrite* (Kawamata et al., 1994) and the compound was identical to trigonelline, which belongs to the betaine group. Our previous study showed that the different organic extracts of the soft coral *Dendronephthya* sp. inhibited the growth and attachment of bacteria from natural biofilm on rocks and that the species composition of the bacteria on the surface of the coral was different from that on inanimate surface (Harder et al., 2003). Three out of eleven bacterial strains isolated from the surface of the *Dendronephthya* sp. suppressed the growth of indigenous bacteria from natural biofilm. All bacterial isolates associated with the surface of the soft coral were insensitive to coral extracts. These findings suggest that this soft coral may directly inhibit larval settlement by releasing antifouling compounds or indirectly inhibit larval settlement by regulating the growth of some bacteria on the coral surface, which in turn affects larval attachment. Thus, we examined the effect of bacterial isolates on larval settlement of common foulers in this study.

Although the culture-dependent technique often underestimates bacterial diversity in environmental samples, this technique was used in this study because the investigation of the effect of bacterial film on larval attachment requires pure bacterial strains for development of bacterial films. The larval settlement bioassay on monospecies bacterial films showed that different strains caused different levels of *H. elegans* larval settlement. Two of the eleven bacterial isolates were ''inhibitive'', four were ''inductive'' and five were ''non-inductive''. In most cases, the larval settlement of *H. elegans* on the bacterial films of pure isolates were lower than that on the NBF (positive control), indicating a possible protective role of the coral associated bacteria. Two bacterial strains (C2 and C8) significantly ($P < 0.05$) suppressed the larval settlement of *H. elegans* and *B. neritina*, while four other bacterial strains caused high percentage of *H. elegans* larval settlement. It may be possible that the role of the inhibitive bacteria overrides the effect of the inductive bacterial strains by their relative abundance or active products and *vies versa*. However, at this moment, it is very difficult to quantify the bacterial abundance of individual species on the surface of the soft coral. Our results indicated that the inhibitive bacteria might indeed counteract the role of the inductive bacteria in this soft coral (see below). The ''inductive'' bacteria on the soft coral surface may play other important roles for the corals. For instance, nitrogen-fixing bacteria provide the host coral *Favia favulus* with additional amounts of nitrogen (Shashar et al., 1994) while symbiotic bacteria from sponges furnish the host organism with fixed carbon or nitrogen (Sara et al., 1998).

In the experiment with the multispecies biofilm, we tried to mimic the microbial community that might be formed on the surface of the soft coral *Dendronephthya* sp. in situ. However, since we do not know the relative abundance of individual bacterial species on the surface of the soft coral, we used an arbitrary equal ratio of cultivable bacterial isolates. When all ''inductive'', ''non-inductive'' and ''inhibitive'' bacterial isolates were mixed, the effect of the resultant biofilm on larval settlement became ''inhibitive''. One may speculate that a natural biofilm on the surface of *Dendronephthya* sp. suppresses larval settlement. Similar results have been demonstrated in an earlier investigation (Lau and Qian, 1997) in which two bacterial strains causing different levels of metamorphosis of *H. elegans* were mixed, and the combined effects became antagonistic, depending on both bacterial species mixed and the ratio of mixture.

In the present study, there was no clear correlation between the activity of bacterial isolates on larval settlement and their phylogenetic origin. For example, strains C3 and C9 genes are very similar to the most ''inhibitive'' strains, C2 and C8 (GenBank) (Table 1), invoked moderate and high larval settlement rates of *H. elegans*, respectively. Generally, the ''inhibitive'' bacterial strains were affiliated with the γ- and α-Proteobacteria phylogenetic branches, while the ''inductive'' or ''non-inductive'' bacterial isolates belonged to *Cytophaga–Flexibacter–Bacteroides*, γ- and α-Proteobacteria phylogenetic branches. Similar results were reported in previous studies (Lau et al., 2002; Lee and Qian, 2003; Pratixa et al., 2003). We therefore conclude that the antifouling activity of bacteria is not related to specific phylogenetic groups.

In the present investigation, we also found that *Vibirio* sp. (C2) and the unidentified α-*Proteobacterium* (C8) significantly suppress larval settlement. CSW from these bacteria inhibited larval settlement of *B. neritina* and *H. elegans*. The compounds with molecular weight greater than 100 kDa from CSW of *Vibirio* sp. (C2) and unidentified α-

Proteobacterium (C8) were highly effective at inhibiting larval settlement. In all experiments, unsettled larvae were alive but could settle only after being transferred into dishes with AFSW from the testing solution, indicating that antifouling compounds from the C2 and C8 strains were nontoxic to larvae.

A biochemical analysis of the CSW showed that the glucose equivalent amount of carbohydrates in the cutoff fractions of the 100 kDa residues was at least six times higher than the bovine albumen amount of proteins. At the same time, the amount of proteins in the 100 kDa cutoff fraction of C2 was two times higher than the one in the 100 kDa retentate of C8. Since biochemical analysis of CSW from C2 and C8 was positive for proteins and carbohydrates, different enzymes were used as probes for the structural correlation of the macromolecular constituents of CSW with their potential biological activity. We observed that *B. neritina* larvae settled and metamorphosed in the control (AFSW and enzymes), indicating that the capacity of larval settlement was not affected by the enzyme probes and that the inhibitory factors to larval settlement in greater than 100 kDa fractions of CSW were indeed the substrates in the digestion experiments. In spite of different carbohydrates and protein composition in the CSW from two bacterial species, the inhibitory compounds demonstrated similar responses to the proteolytic and glycolytic enzyme probes. The inhibitive compounds were indifferent to digestion by papin, trypsin (cleaves specifically proteins) and sulfatase (cleaves organic sulfate esters), whereas after cleavage by α-amylase (digest sugars) the compounds lost their activity. These results suggested that inhibitory compounds from the C2 and C8 strains were large water-soluble polysaccharides. This was further supported by the indifference of CSW activity to the heat-denaturing process.

Marine bacteria associated with basibionts can protect hosts from micro- and macrofouling (Holmström et al., 1992; Holmström and Kjelleberg, 1999; Steinberg et al., 2002). For example, marine bacterium *P. tunicata* isolated from the surface of the tunicate *C. intestinalis* and alga *U. lactuca* produce several extracellular metabolites, ranging from a small (<10 kDa) water-soluble, heat-stable compound to a large protein (190 kDa), which individually inhibit the attachment and growth of bacteria, fungi, algal spores, or invertebrate larvae (James et al., 1996; Holmström and Kjelleberg, 1999; Egan et al., 2000, 2001; Holmström et al., 2002). Epibiotic bacteria *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis* and *Pseudoalteromonas* sp. isolated from different host organisms as well as their supernatants incorporated in sea paints were able to inhibited micro- and macrofouling (Burgess et al., 2003). Similar results have been demonstrated in our previous experiments. The bacteria *Micrococcus* sp., *Micrococcus kristinae*, *Kocuria rhizophila*, *Tenacibaculum mesophilum*, *Pesudoalteromonas piscicida*, *Microbulbifer hydrolyticus* and *α-Proteobacterium* associated with the sponge *Mycale adherens* were able to suppressed larval settlement of the tubeworm *H. elegans* (Lee and Qian, 2003). We found that bacterial strains *Pseudoalteromonas* sp. and *Vibrio* sp. from the seaweed *U. reticulata* inhibited the settlement of *H. elegans* and the growth of the benthic diatom *N. paleacea* (Dobretsov and Qian, 2002). We demonstrated that the putative water-soluble antifouling compound from *Vibrio* sp. is a polysaccharide with molecular weight greater than 100 kDa. Collectively, these findings demonstrate that inhibitory compounds excreted from bacteria are water-soluble proteins or carbohydrates. In addition, it is proposed that, in contrast to inducers, deterrents appear to be primary nonpolar, low soluble secondary

metabolites effective at low concentrations (Steinberg et al., 2001, 2002). One possible reason of these contradictory results could be that antifouling compounds, especially large carbohydrates, produced by bacteria mainly accumulate in the bacterial film matrix and released in water at low concentrations.

How can carbohydrates from *Vibirio* sp. (C2) and unidentified α-*Proteobacterium* (C8) inhibit larval settlement? It has been demonstrated that polysaccharides in a variety of biological systems are mediated by lectins (Morse and Morse, 1991). The attachment and metamorphosis of the polychaete *Janua brasiliensis* are mediated by a lectin-like recognition of inductive exopolysaccharides produced by the specific bacteria *Pseudomonas marina* (Kirchman et al., 1982; Mitchell and Kirchman, 1984; Maki and Mitchell, 1985). Similar results have been shown for hydrozoan and barnacle larvae (Matsumura et al., 1998; Railkin and Chikadze, 1999; Khandeparker et al., 2002, 2003). In these experiments, lectins or sugars effectively inhibited larval settlement. Therefore, we speculate that carbohydrates produced by bacteria could bind to the lectin receptors of larvae and block larval attachment.

In conclusion, this study demonstrated that two coral associated bacteria, *Vibrio* sp. (C2) and unidentified α-*Proteobacterium* (C8), could suppress the settlement of *H. elegans* and *B. neritina* larvae. Antifouling compounds from these bacteria were water-soluble, heat-stable polysaccharides with a molecular weight greater than 100 kDa. However, the main role of coral associated "inductive" bacteria, the proportion of "inhibitive" bacteria on the surface of soft coral in contrast to "non-inhibitive" bacteria, and interaction between inhibitive compounds from the soft coral and from the coral-associated bacteria remain to be investigated in future studies.

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