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MARINE BIOTECHNOLOGY

Novel Antifoulants: Inhibition of Larval Attachment by Proteases

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Abstract

We investigated the effect of commercially available enzymes (α-amylase, α-galactosidase, papain, trypsin, and lipase) as well as proteases from deep-sea bacteria on the larval attachment of the bryozoan *Bugula neritina* L. The 50% effective concentrations (EC_{50}) of the commercial proteases were 10 times lower than those of other enzymes. Crude proteases from six deep-sea *Pseudoalteromonas* species significantly decreased larval attachment at concentrations of 0.03 to 1 mIU ml^{-1}. The EC_{50} of the pure protease from the bacterium *Pseudoalteromonas issachenkonii* UST041101-043 was close to 1 ng ml^{-1} (0.1 mIU ml^{-1}). The protease and trypsin individually incorporated in a water-soluble paint matrix inhibited biofouling in a field experiment. There are certain correlations between production of proteases by bacterial films and inhibition of larval attachment. None of the bacteria with biofilms that induced attachment of *B. neritina* produced proteolytic enzymes, whereas most of the bacteria that formed inhibitive biofilms produced proteases. Our investigation demonstrated the potential use of proteolytic enzymes for antifouling defense.

Keywords: antifouling — deep-sea bacteria — enzymes — larval attachment — proteases — settlement

Introduction

Natural and artificial substrates in the marine environment are quickly colonized by microfoulers (marine bacteria, protozoans, and microalgae) and macrofoulers (invertebrates and macroalgae) (Wahl, 1989; Little and Wagner, 1997; Railkin, 2004). Both micro- and macrofouling in the world's oceans cause huge material and economic losses in maintenance of mariculture facilities, shipping facilities, vessels, and seawater pipelines (Wahl, 1997; Clare, 1998; Fusetani, 2004; Yebra et al., 2004). Worldwide, countries spend more than US$5.7 billion annually to prevent and control marine biofouling (Rouhi, 1998). To minimize the impacts of foulers, many underwater structures are protected by antifouling coatings. Yet, these coatings are highly toxic and nonspecific (Yebra et al., 2004). As an example, it has been shown that extremely low concentrations of tributyltin (TBT) (1 to 20 ng l^{-1})—the mostly common antifouling agent—cause defective shell growth in the oyster *Crassostrea gigas* and development of male characteristics in female genitalia in the dogwhelk *Nucella* sp. (Yebra et al., 2004). The ban of TBT and other toxic biocides in marine coatings (van Wezel and van Wlaardingen, 2004) has created a need for the shipping industry and the producers of coatings to develop alternative technologies to prevent fouling on ship hulls.

Marine biofilms can enhance (Kirchman et al., 1982; Maki et al., 1988; Lau and Qian, 2001) or inhibit larval settlement (Maki et al., 1988; Holmström et al., 1992, 2002; Egan et al., 2001, 2002; Dobretsov and Qian, 2002, 2004; Lau et al., 2003). It has been shown that bacterial chemical compounds inhibit larval settlement and may be used as "environmentally friendly" antifoulants for protection against marine biofouling (Holmström and Kjelleberg, 1999; Burgess et al., 2003). Moreover, bacterial strains can be cultivated in large amounts and produce diverse chemical compounds under optimal conditions. All these factors make bacteria an important source of antifouling compounds (Dobretsov et al., 2006). Compared to the large number of antifouling compounds that have been reported from marine macroorganisms, only few antifouling compounds have been isolated from marine bacteria (Fusetani, 2004). For example, the marine bacterium *Alteromonas* sp. produced the

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antifouling compound ubiquinone-8 (Kon-ya et al., 1995) that inhibited settlement of the barnacle *B.amphitrite* at concentrations of 12.5 to 25.0 ppm.

Many biofouling species, such as bacteria, diatoms, algal spores, and invertebrate larvae use protein and glycoprotein polymers for their attachment to the surfaces (Cooksey and Wigglesworth-Cooksey, 1995; Callow and Callow, 2000; Kamino, 2001). Algal spores use polysaccharides, proteins, and their complexes for spores attachment and adhesion (Vreeland et al., 1998). In contrast, the adults of the mussel *Mytilus edulis* use proteins, which mostly contain the amino acid 3,4-dihydroxyphenylalanine (DOPA) for their attachment (Waite, 1992; Wiegemann, 2005). Similarly, larvae of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina* attach to substrata via different proteins (Matsumura et al., 1998; Kamino, 2001; Railkin, 2004). Therefore, we hypothesized that enzymes that cleave proteins and carbohydrates can inhibit larval attachment of the bryozoan *Bugula neritina*—one of the major foulers in tropical waters. In addition, we tested the effects of sulfatases and lipases as many of adhesive proteins and carbohydrates may contain sulfate esters and fatty acid groups. We tested our hypothesis by addressing the following questions: (1) Do commercially available enzymes (proteases, sulfatases, lipases, and saccharidases) inhibit larval attachment of *B. neritina*? (2) Do proteolytic enzymes produced by deep-sea bacteria inhibit larval attachment in laboratory and field experiments? (3) Is there a correlation between the production of proteolytic enzymes by bacterial isolates and the inhibition of larval attachment on the corresponding bacterial biofilms?

Materials and Methods

Commercially Available Enzymes. For larval bioassay experiments we use the following enzymes: proteases—trypsin (from bovine pancreas, T1426) and papain (from *Carica papaya*, no. 76220); sulfatases—sulfatase (from *Helix pomatia*, S9626); lipases—lipase (from *Candida rugosa,* L1754); and saccharidases—α-amylase (from *Aspergillus oryzae*, A6211) and β-galactosidase (from *Escherichia coli*, no. 48275). All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). Solutions of the enzymes were prepared with sterile filtered (0.22 μm) sea water (FSW) and concentrations from 4 mIU ml^{-1} to 0.04 mIU ml^{-1} were used in the experiments. The enzymatic activity (i.e., proteolytic, glycolytic, etc.) was calculated by the multiplication of enzyme weight and its commercial specific activities. The concentration of enzymes was expressed as mIU ml^{-1} because the concentration of enzymes in weight per volume depends on chemical purity of compounds and cannot accurately describe their enzymatic activity. In addition, for comparison with other reports, the concentrations of enzymes were expressed as ng ml^{-1}.

Isolation of Bacteria. Sediment samples were collected from three deep-sea locations near Unimak Island along Aleutian margin, Pacific Ocean. Sediments were removed from the upper 6 cm of tube cores collected using the remotely operated vehicle JASON II on a cruise aboard the R/V Revelle in July, 2004. The strains D1-001, D2-005, and D3-103 were collected from 53°27′N, 163°22′W, at a depth of 4240 m; the strain D4-001 was collected from 53°30′N, 163°27′W, at a depth of 3283 m and the strains D12-004 and D12-006 were collected from 53°30′N, 163°26′W, at a depth of 3310 m. For bacterial isolation the sediments were placed into sterile test tubes, kept cool (4 to 6°C), and transported to the laboratory of the Hong Kong University of Science and Technology (HKUST). To isolate bacteria, 1 g of sediment was resuspended in 10 ml of filtered (0.22 μm) seawater. Aliquots of 200 μl of each suspension were streaked onto nutrient agar (0.3% yeast extract, 0.5% peptone, 1.5% agar, FSW) with replication ($n = 3$) and incubated at 15 ± 1°C under a 15 h light:9-h dark photoperiod under normal pressure conditions. After 1 to 3 days of incubation, bacterial colonies were examined for conspicuous characteristics such as color, shape, size, surface topography, and the presence of granules under a dissecting microscope. Distinguishable colonies were isolated and purified.

In addition, bacterial strains were isolated from 7-day-old natural biofilm from the *B. neritina* habitat (Port Shelter Bay, 22°21′N, 114°16′E). The bacterial strains were isolated and identified by comparative analysis of their 16S rRNA gene sequences as described in Lau et al. (2002). The closest match to the 16S rRNA gene sequence of the respective bacterium was retrieved by comparison with data from GenBank (http://www.ncbi.nlm.nih.gov). The bacterial isolates were stored in 50% glycerol at –80°C unless needed for the experiment.

The proteolytic activity of bacterial cultures was screened on a skim-milk agar medium containing 2% of skim-milk powder and 1% of tryptone (wt/vol). After 1 week of cultivation at room temperature (23°C), a colony showing a clear zone indicated which bacteria showed higher protease activity. Six bacterial strains (D1-001, D2-005, D3-103, D4-001, D12-004, and D12-006) exhibited the strongest protease activity and were chosen for the subsequent laboratory experiments.

Production, Isolation, and Purification of Enzymes from Bacteria. Strains were grown in marine 2216 broth (Difco Laboratories, Fisher Scientific, Pittsburgh, PA) at 4°C in 2-liter shake flasks (150 rpm). After 5 days the cell pellets were separated from the medium by centrifugation (5000*g*). Ammonium sulfate (Sigma-Aldrich) was added to the spent culture media to achieve 80% saturation. The suspension was centrifuged (5000*g*) and the precipitate was dissolved into 25% saturated ammonium sulfate in 50 mM Tris-HCl (pH 7.5). Six of the crude bacterial supernatants with enzymatic activity were dialyzed overnight (Spectrum Laboratories Inc., Laguna Hills, CA, MWCO 1000) to remove ammonium sulfate for the larval settlement bioassay. The protease produced by the strain D12-004 (*Pseudoalteromonas issachenkonii* UST041101-043, GenBank accession no. DQ178021) that has the strongest antifouling effect was purified by methods described previously (Xiong et al., 2004, 2007). Briefly stated, the sample was applied to a phenyl Sepharose® column (Amersham Pharmacia Biotech, Uppsala, Sweden) and was eluted with a linear gradient of 25 to 0% saturated ammonium sulfate. After ultrafiltration through 1-kDa membrane to remove the salts, the active fraction was applied to a DEAE Sepharose® (Amersham Pharmacia Biotech, Uppsala, Sweden) ion-exchange column and eluted using a linear gradient of 0 to 1 M sodium chloride solution with buffer. The purified enzyme was stored at −20°C in 20 mM ammonium acetate buffer (pH 6.0).

Protease Activity Detection and Measurements. The filter paper diffusion experiment was designed to detect protease activity. A skim-milk agar plate containing 2% of skim-milk powder and 1.5% agar was the detection medium. A piece of filter paper disk (diameter of 4 mm) with 50 μl of suspected sample was laid on the plate. After a 1-day incubation at room temperature (23°C), the transparent zone indicated the protease activity.

Protease activity was measured by assaying the UV absorbance of the liberated amino acids using casein as a substrate (Ghorbel et al., 2003; Xiong et al., 2004). Suitably diluted enzyme solution (0.5 ml) was mixed with 0.5 ml of buffer (50 mM Tris-HCl, pH 7.5) containing 0.5% casein, and incubated for 30 min at 35°C. The reaction was stopped by addition of 0.5 ml of trichloroacetic acid (20%, wt/vol). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 13,000 rpm for 15 min to remove the precipitate. The absorbance of supernatant was measured at 280 nm. A standard curve was generated using solutions of 0 to 50 mg l^{-1} tyrosine. One unit (IU) of protease activity was defined as the amount of enzyme that liberated 1 micromole of tyrosine in 1 min. All experimental data are given as the mean of three independent experiments with three replicates, unless otherwise stated.

Larval Culture. Adults of the bryozoan *Bugula neritina*, were collected from submerged rafts at the fish farms in Yung Shue O, Hong Kong (114°21′E, 22°24′N). Larvae were obtained according to the method described by Dobretsov and Qian (2004); and only newly released larvae (i.e., within 10 min) were included in the bioassays.

Larval Bioassays. The larval bioassay was performed several times on different larval batches with replication ($n = 6$) in sterile polystyrene 24-multiwell plates (Falcon, Brookings, SD). Each well contained around 20 larvae of *B. neritina* and the enzyme under investigation. In experiments with bacterial films, we used Petri dishes (no. 1006, Falcon) biofilmed according to Dobretsov and Qian (2006). The choice bioassay was conducted in double-sided test containers. One dish contained biofilms and the other dish was sterile, so that larvae in each container would have a choice of two surface types for attachment. Twenty larvae were introduced in each dish containing monospecies bacterial films. In all bioassays, FSW were used as a control. Purified protease and trypsin solution heated at 100°C for 15 min were used as additional controls in some experiments. Larval settlement assays were run at 23°C under continuous illumination for 1 h. After the testing period, the number of metamorphosed and swimming larvae was counted with a microscope.

Field Experiments. Pure enzymes, such as trypsin (from bovine pancreas, T1426, Sigma) and protease E1 from the deep-sea bacterium *P. issachenkonii* UST041101-043, were dissolved in distilled water. Trypsin and the protease were individually mixed with water-based vinyl paint, MC1111 (Macpherson Ltd.) to obtain concentrations of 10 mIU ml^{-1}, 100 mIU ml^{-1}, and 250 mIU ml^{-1}. In field experiments trypsin was tested at concentrations of 80 ng ml^{-1}, 0.8 μg ml^{-1}, and 2 μg ml^{-1}, while the protease was tested at concentrations 100 ng ml^{-1}, 1 μg ml^{-1}, and 2.5 μg ml^{-1}. The proteolytic activities of trypsin and protease were measured by hydrolysis of casein at 35°C (pH 8). All prepared paints were applied by brushes to plastic panels (10×10 cm, $n = 3$) and air dried for 24 h before the experiment. Distilled water added to the vinyl paint and deactivated by heating enzymes mixed with the paint was used as a positive control. An antifouling paint Interspeed Ultra (International Ltd.), which contains the biocides copper oxide and preventol A4S, was

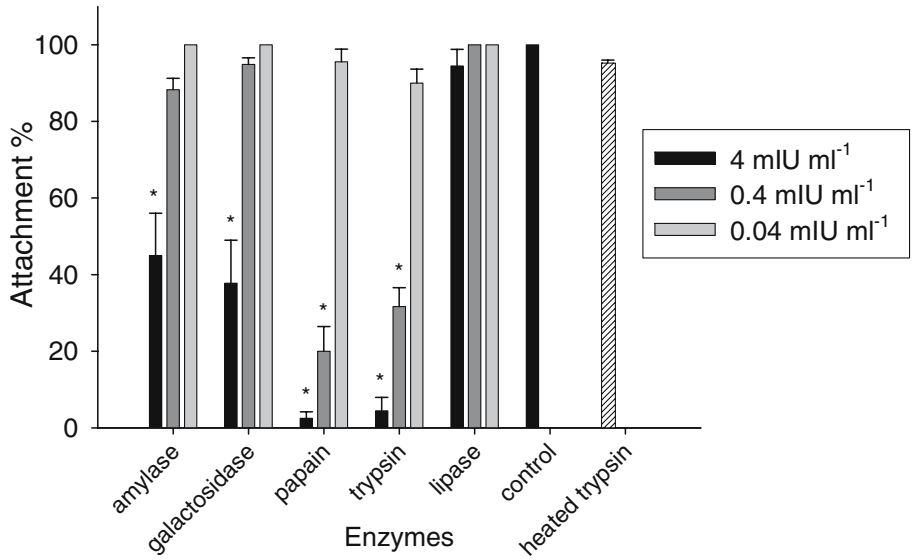

Figure 1. Antifouling effect of commercially available enzymes on larval settlement of *Bugula neritina*. Larval settlement was calculated based on the number of larvae settled 1 h after the commencement of bioassay. Filtered sea water (FSW) and heated (100°C) trypsin were used as controls. Data are the means + 1 SD. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

used as a negative control. Panels were exposed to biofouling at a depth of 1 m at the fish farm Yung Shue O, Hong Kong (114°21′E, 22°24′N) for 14 days in December, 2005. Seawater temperature during the experiments was 20°C and salinity was 35‰.

Statistical Analysis. The numbers of attached larvae were converted into percentages that were then arcsine-transformed. In the case of zero attached larvae, a value of 4 n^{-1} (n = number of larvae in a single replicate) was assigned to improve the arcsine transformation (Zar, 1996). The normality assumption was verified via the Shapiro-Wilk test (Shapiro and Wilk, 1965). The density of the bacteria was square-root transformed to ensure normality of variance. The differences between bacterial densities and larval attachment in the laboratory and field experiments with enzymes were analyzed with one-way ANOVA followed by a Dunnet post hoc test. In addition, attachment in test and control vessels with monospecies biofilms was compared to the null hypothesis of 50:50 distributions of attached larvae on either side of the vessel, using a replicated *G*-test for the goodness of fit (Zar, 1996). The *G*-value was calculated as a measure of heterogeneity among replicate vessels within experimental type, as well as among experimental replicates. Homogeneous data sets were pooled and corresponding

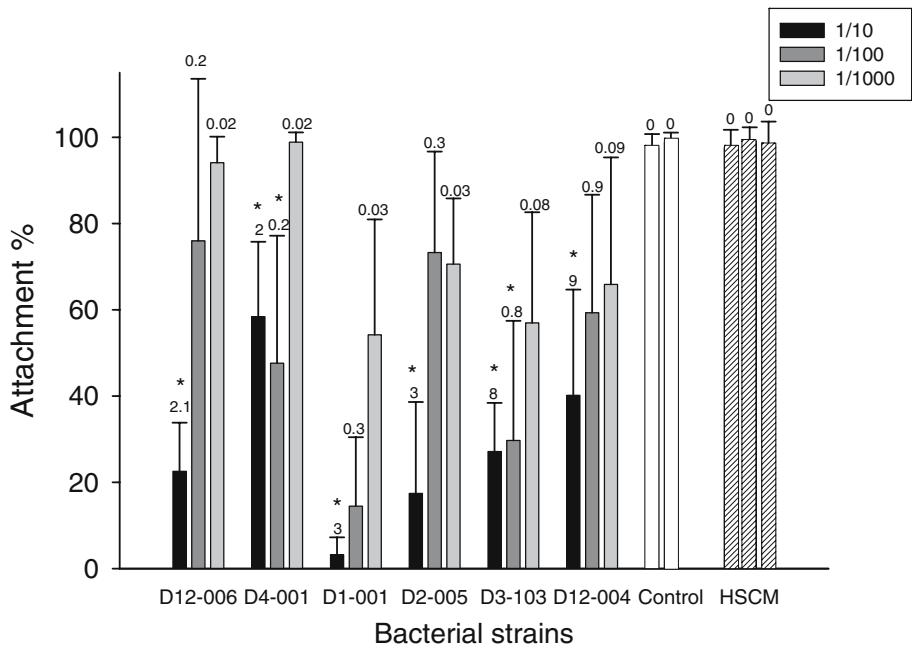

Figure 2. Effect of spent culture medium from six bacteria isolated from the deep sea sediments on larval attachment of *Bugula neritina*. Autoclaved FSW and heated spent culture mediums (HSCM, 100°C) were used as controls. Before the experiments, spent culture medium was diluted 10, 100, and 1000 times. The spent culture media protease activities (mIU ml^{-1}) of the tested bacteria are shown above the bars. Bars are the means of six replicates + 1 SD. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

Table 1. Coding of deep-sea bacterial isolates used for the experiments

Close matched species	*GenBank accession no.*	*Similarity according to NCBI (%)*	*Isolated from location*	*Strain code*
Pseudoalteromonas paragorgicola	AY040229	97	53°27′N, 163°22′W,4240 m	D1-001
Pseudoalteromonas tetraodonis	AF214729	99	53°27′N, 163°22′W,4240 m	D2-005
Pseudoalteromonas sp. UST020129-019	AY241428	99	53°27′N, 163°22′W,4240 m	D3-103
Pseudoalteromonas tetraodonis	AF214729	99	53°30′N, 163°27′W,3283 m	D4-001
Pseudoalteromonas issachenkonii UST041101-043	AF316144	98	53°17′N, 163°26′W,3310 m	D12-004
Pseudoalteromonas sp. MGP-2	AF530129	99	53°17′N, 163°26′W,3310 m	D12-006

G-values were transformed by Willam's correction (Zar, 1996). In all cases, the threshold for significance was 5%. The data presented in all the figures herewith are not transformed.

Results and Discussion

At high concentrations, all tested enzymes except lipase inhibited larval attachment (Figure 1). At concentrations of 0.4 mIU ml^{-1} (about 3 ng ml^{-1}), only proteolytic enzymes (papain and trypsin) but not glycolytic and lipolytic enzymes significantly ($P < 0.05$) inhibited larval settlement. At concentrations of 0.04 mIU ml^{-1} (below 0.3 ng ml^{-1}) all tested enzymes had no effect on larval attachment. Heated trypsin did not show any antifouling activity. These results suggested that proteolytic activity of trypsin is responsible for antifouling effect. The enzymes did not affect larval survival, and after larvae were washed with fresh seawater, they settled (data not shown).

The taxonomic identification showed that all of deep-sea bacterial isolates that produced proteases belonged to the genus *Pseudoalteromonas* sp. (Table 1). Spent culture medium from all *Pseudolateromonas* isolates significantly inhibited larval settlement of *B. neritina* ($P < 0.05$) compared to the control (FSW) (Figure 2). Heated spent culture medium (HSCM) lost its proteolytic activity and did not affect larval attachment, suggesting that proteins and possibly enzymes are responsible for inhibition of larval attachment. Spent culture medium from deep-sea bacteria (Table 1) was further purified and crude enzymes (precipitated with ammonium sulfate and dialyzed) were tested against larval settlement of *B. neritina* (Figure 3). At high concentrations (1 mIU ml^{-1} and 0.2 mIU ml^{-1}), all crude enzymes significantly ($P < 0.05$) decreased larval attachment. At concentrations of 0.03 mIU ml^{-1} and 0.06 mIU ml^{-1}, the crude enzymes did not significantly change larval attachment, except for the crude enzyme from the strain D12-004. The larvae remained alive in the crude enzymes solutions and the effect of crude enzymes was reversible.

The proteases from deep-sea bacteria can be divided into two groups according to the optimum pH values of the protease activity (Xiong et al.,

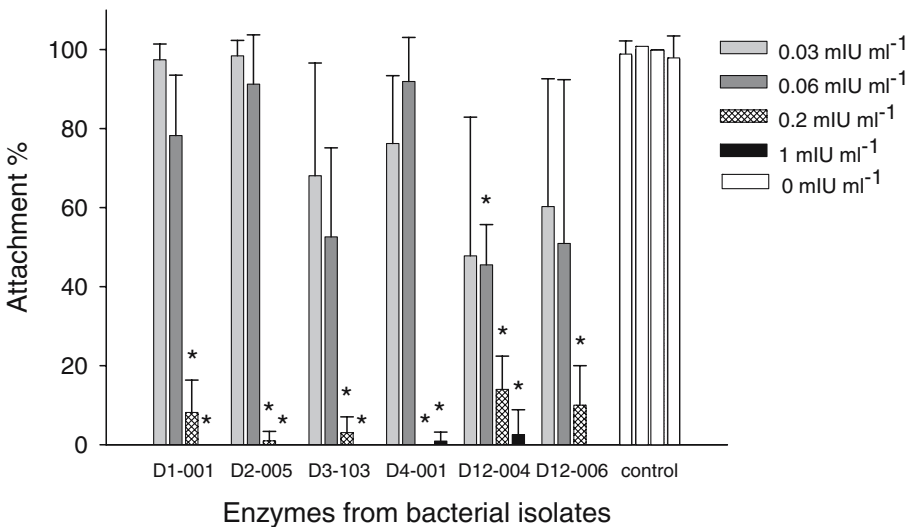

Figure 3. Effect of crude enzymes (see Materials and Methods) from deep-sea bacteria on larval settlement of *Bugula neritina.* Autoclaved FSW was used as a control. Concentration of enzymes: gray = 0.03 mIU ml^{-1}; dark gray = 0.06 mIU ml^{-1}; stipled = 0.2 mIU ml^{-1}; black = 1 mIU ml^{-1}; white = 0 mIU ml^{-1} (FSW). Bars are the means of six replicates + 1 SD. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

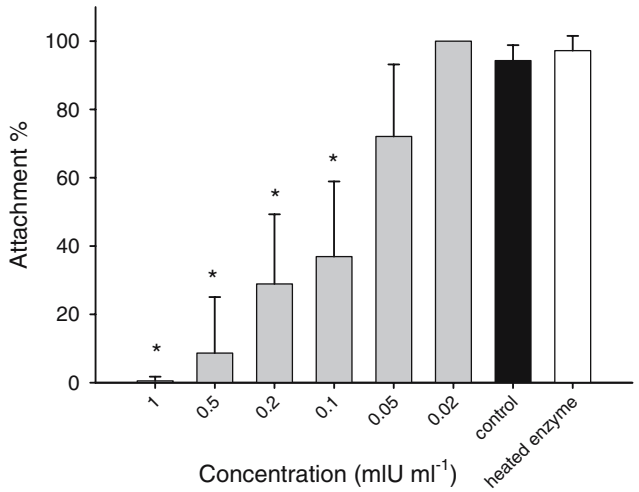

Figure 4. Effect of the purified protease from *Pseudoalteromonas issachenkonii* UST041101-043 (D12-004) on larval settlement of *Bugula neritina*. The mixture of autoclaved FSW and deactivated 1 mIU ml^{-1} protease (enzyme heated until 100°C) were used as controls. Bars are the means of six replicates + 1 SD. Data that are significantly different from the control according to a Dunnet test (ANOVA: $P < 0.05$) are indicated by an asterisk above the bars.

2007). The optimum pH range of the proteases from the strains D12-004 and D3-103 was pH 7 to 8, and optimal activity observed at temperature close to 30°C, whereas the optimum pH of the proteases D2-005, D4-001, D1-001, and D12-006 was close to pH 9 and they were active at temperatures of 40 to 45°C. Our data suggest that some of bacterial enzymes have optimal performance at conditions close to that in seawater and these enzymes can be used for antifouling applications.

One deep-sea bacterial strain (*Pseudoalteromonas issachenkonii* UST041101-043; D12-004) that demonstrated the highest antifouling activity (Figure 3) and showed maximal activity at pH and temperature range typical for tropical waters (Xiong et al., 2007) was selected for the further purification of protease. Xiong et al. (2007) described purification of neutral, cold-adapted protease from *P. issachenkonii*; therefore we present only results of bioactivity of this protease. Protease with activity above 0.1 mIU ml^{-1} (above 1 ng ml^{-1}) significantly decreased larval attachment (Figure 4). After heat denaturation of the enzyme, antifouling activity was lost. This means that the enzyme caused antifouling activity of *P. issachenkonii*. The attachment of *B. neritina* in dishes with enzymes at 0.05 mIUml^{-1} (0.5 ng ml^{-1}) was lower than that in the FSW (control), but not significantly. The effective concentration of protease for 50% inhibition of larval settlement (EC_{50}) was 0.1 mIU ml^{-1}, which is equal to 1 ng ml^{-1}. This concentration is much lower than that of other antifouling compounds (Dobretsov et al., 2006). Therefore, the proteolytic enzyme from *Pseudoalteromonas issachenkonii* UST041101-043 has great potential as a future antifouling agent in tropical waters.

Only limited information about the effect of enzymes on larval settlement is available from the literature. Callow et al. (2000) reported that trypsin, proteases A and B cleave adhesive of spores of the alga *Enteromorpha* sp. and thus prevent their attachment. The serine proteases were found to have the broadest antifouling effect against adhesion of spores of *Ulva linza*, cells of the diatom *Navicula perminuta*, and cyprids of the barnacle *Balanus amphitrite* (Pettitt et al., 2004). The antifouling effects were observed at a minimal enzyme concentration of 0.44 μg ml^{-1}, which is more than

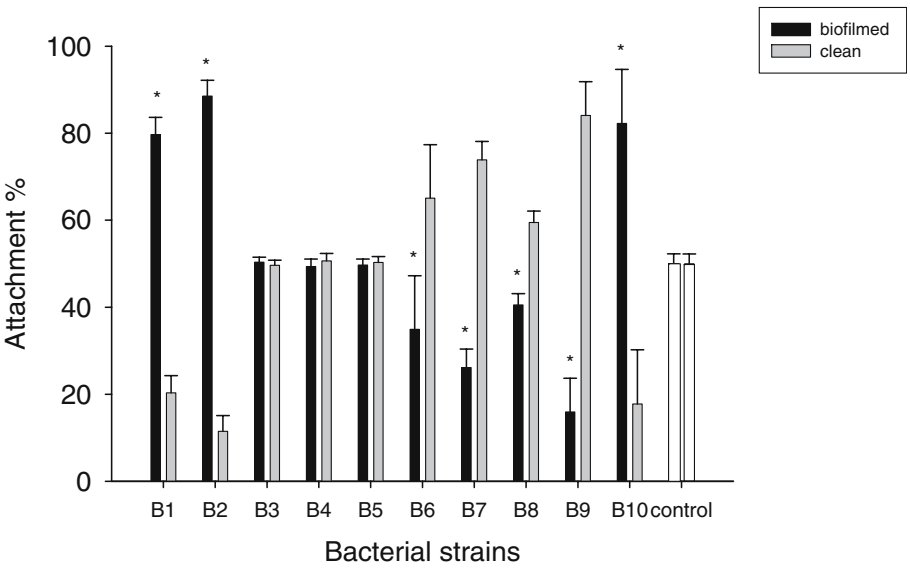

Figure 5. Effect of monospecific bacterial films on larval attachment of *Bugula neritina*. Each test vessel contained biofilmed and sterile (unfilmed) dishes. Two sterile dishes were used as a control. Significantly different (*G*-test, $P < 0.05$) settlement on biofilmed dishes is indicated by an asterisk.

400-fold higher than in our experiments. After inactivation of enzymes by heating, the antifouling effect remained, suggesting that non-enzymatic components in the enzyme formulations played a role in the inhibition of biofouling. Therefore, the results of Pettitt et al. (2004) need to be interpreted with caution. In a series of patents, inventors proposed to use the enzymes to prevent biofouling (Selvig et al., 1999; Bonaventura et al., 2000; Allermann and Schneider, 2001). For example, proteolytic and amylolytic enzymes inhibited recruitment of algal spores and a bryozoan, and attachment of bacteria, but did not inhibit barnacle settlement (Selvig et al., 1999). Rittschof et al. (1991) investigated the effect of commercially available hydrolytic enzymes on the barnacle *Balanus amphitrite* settlement. The majority of enzymes did not have any effect on larval settlement at concentrations ranging from 1 to 100 nM. The investigators observed that only protease XI at the concentration of 100 nM (about 2 mg ml^{-1}) inhibited larval settlement, while 10 nM of trypsin (about 238 μg ml^{-1}) stimulated larval settlement. The difference between our results and those of the investigation of Rittschof et al. (1991) may be explained by the difference in larval response to enzymes and the concentrations of enzymes used.

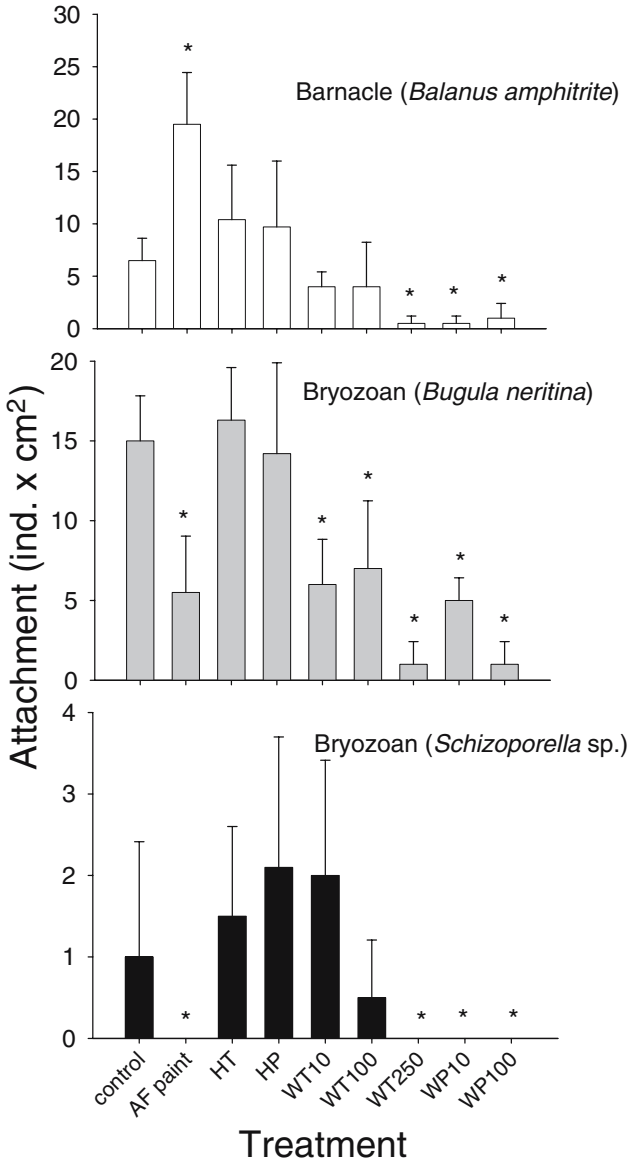

Figure 6. Effect of trypsin (WT) and protease from *Pseudoalteromonas issachenkonii* UST041101-043 (WP) incorporated in water-based paints on biofouling. The numbers correspond to the mIU ml^{-1} of enzyme added to the paint. The paint with distilled water added and the paint with heated enzymes [HT (trypsin) and HP (protease)] at 100 mIU ml^{-1} were used as controls. AF paint is a biocidal antifouling paint (International Ltd). Bars show the means of three replicates + 1 SD. Data that are significantly different from the control (ANOVA, Dunnet test: $P < 0.05$) are indicated by an asterisk above the bars.

How can enzymes decrease larval attachment? There are two possible explanations. First, to attach to the surface, larvae, spores of algae, and microorganisms use different adhesive polymers (Callow and Callow, 2000; Wiegemann, 2005). For example, bacteria and algae use adhesive proteins, polysaccharides, and glycoproteins for adhesion (Becker, 1996; Vreeland et al., 1998). Proteins with high DOPA content play an important role in the adhesion of bivalve molluscs, such as *Mytilus edulis* (Waite, 1992; Wiegemann, 2005). The barnacle larvae and bryozoans achieve adhesion by secretion of proteinaceous cement (Kamino, 2001; Matsumura et al., 1998; Railkin, 2004). Silk-like proteins enriched with DOPA are used for tube construction by the polychaete *Phragmatopoma californica* (Jensen and Morse, 1988). Because most adhesive substances consist of proteins and carbohydrates, we propose that proteolytic or glycolytic enzymes can cleave adhesives of larvae and algal spores and therefore prevent attachment of propagules. Second, enzymes can remove proteinaceous cues necessary for *Bugula neritina* attachment. Although inductive cues for *B.neritina* have not been found yet (Dobretsov and Qian, 2006), other larvae, like barnacles, have been shown to respond to proteinaceous cues produced by adults (Clare et al., 1994; Matsumura et al., 1998).

In our experiments, most of the bacterial isolates that induced larval settlement of *B. neritina* did not produce proteolytic enzymes (Table 2; Figure 5). In contrast, the strains that produced enzymes either were inhibitive or induced low settlement of larvae. Therefore, we found a strong negative correlation between the effect of bacterial biofilms on larval

Table 2. Correlation between production of proteolytic enzymes and inductiveness of bacterial isolates from biofilms and *Pseudoalteromonas issachenkonii* isolated from deep water

Abbreviation	*Close match*	*UST accession no.*	*Similarity (%)*	*Production of proteolytic enzymes*	*Induction of B. neritina settlement*
α–Proteobacteria					
B1	*Rhodovulum* sp.	UST950701-012	98	–	Inductive
B2	*Agrobacterium altanticum*	UST010801-005	99	–	Inductive
γ-Proteobacteria					
B3	*Pseudoalteromonas* sp.	UST010723-006	97	+	Noninductive
B4	*Vibrio campbelli*	UST991130-066	98	+	Noninductive
B5	*Alteromonas* sp.	UST040911-046	96	+	Noninductive
B6	*Vibrio alginolyticus*	UST981130-062	99	+	Inhibitory
B7	*Vibrio* sp.	UST991130-048	98	+	Inhibitory
B8	*Vibrio proteolyticus*	UST991130-028	95	+	Inhibitory
B9	*Pseudoalteromonas issachenkonii*	UST041101-043	98	+	Inhibitory
Firmicutes					
B10	*Staphylococcus haemolyticus*	UST950701-004	99	–	Inductive

Inductive strains induce significantly high ($P < 0.05$) larval settlement; the settlement rate in noninductive strains is the same as in the control (nonbiofilmed petri dish); inhibitory strains significantly ($P < 0.05$) reduced larval settlement (see Figure 5). –, No enzyme production; +, enzyme production.

settlement and production of proteolytic enzymes, which may suggest that biofilm rejection by larvae may not only result from production of repellent or inhibitive cues by bacteria (Holmström and Kjelleberg, 1999; Egan et al., 2001, 2002; Dobretsov and Qian, 2002, 2004), but also as a result of production of enzymes by bacterial species.

In our field bioassay, the protease from *Pseudoalteromonas issachenkonii* UST041101-043 incorporated in water-based paint significantly inhibited settlement of the barnacle *Balanus amphitrite* and the bryozoans *Bugula neritina* and *Schizoporella* sp. compared to the control (paint without enzyme) or deactivated enzyme (paint with heated enzyme) (Figure 6). Moreover, the inhibitory effect of the protease from the bacterium *P. issachenkonii* was stronger than that of trypsin. For example, a concentration of 250 mIU ml^{-1} (about 2 μg ml^{-1}) trypsin was high enough to suppress recruitment of *B. amphitrite* and *Schizoporella* sp., while the protease from the bacterium was effective at lower concentrations (10 mIU ml^{-1} or about 100 ng ml^{-1}). Antifouling activity of both enzymes against the barnacle *B. amphitrite* was stronger than of the biocidal antifouling paint (Figure 6).

At present, industries control biofouling by using highly toxic antifouling compounds. Suppressing biofouling without the use of biocides that kill propagules can be done by controlling propagules adhesion, either by impeding the production of adhesive or by curtailing the strength of that adhesive once secreted. In the natural environment microorganisms can easily biodegrade enzymes to amino acids and finally to carbon dioxide and water; therefore, it is generally accepted that enzymes are "environment-friendly" agents. Stability of enzymes in seawater and solvent-based paints limits commercial application of enzymes as antifouling agents. It has been shown that some enzymes (i.e., halophilic proteases) are quite stable in organic solvents (Klibanov, 2001). In our experiments, protease from *P. issachenkonnii* remained active in seawater for 14 days at high salt concentrations of about 2 M and in acetone (Xiong et al., 2007). In addition, this enzyme has an optimal temperature (about 30°C) and pH (about 8) close to those observed at tropical waters, while other tested enzymes were effective at different temperature and pH. All these facts suggest that the protease from *P. issachenkonnii* can be used widely for different seawater antifouling applications.

Overall, our investigation demonstrates that proteases can act as antifouling agents. The critical doses for proteases are relatively lower than those for other common antifoulants. In our experiments the minimal effective protease concentration was about 1 mIU ml^{-1}, which means that the final pure protease matter concentration was about 1 ng ml^{-1}. In contrast, the other biocides, such as TBT, had six fold higher effective concentrations (Zentz et al., 2002). Therefore, proteases may be useful in the development of future "environment-friendly" antifouling coatings.

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