The inhibitory effect of biofilms produced by wild bacterial isolates to the larval settlement of the fouling ascidia *Ciona intestinalis* and *Pyura praeputialis*

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Abbreviations: HPLC: high pressure liquid chromatrography
MMM: marine minimal medium
TBT: tributyl-tin

Marine biofouling is a present and potentially increasing future problem at molluscan culture centres. The problem is highly variable, exists on different scales, and its negative impact on cultured organisms and related economic losses at these centres has not been significantly controlled. One approach to fouling control has been the incorporation of natural substances into anti-fouling paints which inhibit the settlement of common fouling organisms. The main objective of the present study was the isolation of naturally occurring substances from marine bacteria which were inhibitory to the settlement of *Ciona intestinalis* and *Pyura praeputialis,* two tunicate species causing serious fouling problems in scallop culture systems in Chile. Numerous bacterial strains were isolated from microfouling on natural and artificial substrates submerged in the sea; of 73 strains isolated, 20% demonstrated inhibitory effects on the settlement of the larvae of the above cited tunicates. The inhibitory substances produced by the active bacteria were extracellular, and could be incorporated in an inert matrix (Phytagel[TM]) without losing their inhibitory properties. Some properties of the inhibitory substance isolated from bacterial strain Clon Nil-LEM (*Alteromonas* sp) included thermostability, MW < 3500 Da, peptidase lability (against *C. intestinalis*), and undiminished inhibitory activity when incorporated in the inert matrix.

Biofouling causes severe problems for structures submerged in the sea, such as increases in mass, and corrosion of surfaces resulting in economic losses. These effects are of particular consequence in the aquaculture industry, and on structures in the sea such as the supports of oil drilling platforms and ship hulls (Armstrong et al.

2000). A common method for avoiding settlement of marine fouling organisms on marine structures has been coating with antifouling paints, preferably those containing tributyl-tin (TBT) or copper. These paints are highly effective in controlling fouling on ships, with periods of up to seven years without maintenance and representing savings of US$ 6.5 billion per year. Considerations of environmental protection, however, now prevent use of the TBT coatings on vessels of under 25 m in length. Numerous studies have been made on the effects of TBT on marine organisms such as the molluscs, in which toxic doses may be as low as 1 ng L^{-1} (Grinwis et al. 1998; Fisher et al. 1999).

Environmentally acceptable antifouling substances are needed for incorporation into antifouling coatings, and these may include natural products isolated from certain marine organisms (Clare, 1996) which natural products isolated from certain marine organisms (Clare, 1996). Incorporation of naturally repellent products into anti-fouling paints has been tried by some researchers (Armstrong et al. 2000; Peppiatt et al. 2000). Some marine organisms such as corals, algae, sponges, and ascidians have been shown to produce antifouling substances which in nature maintain them free from undesirable encrusting organisms (Hentschel et al. 2001; Dobretsov and Qian 2002; Harder et al. 2003).

Some studies have shown that bacterial biofilms isolated from organisms having low degrees of surface macrofouling release compounds which repel other bacteria, acting in a protective role (Boyd et al. 1998; Boyd et al. 1999a; Boyd et al. 1999b; Burgess et al. 2003).Some bacteria present on the surfaces of crustacean larvae produce antimicrobial compounds which protect them from
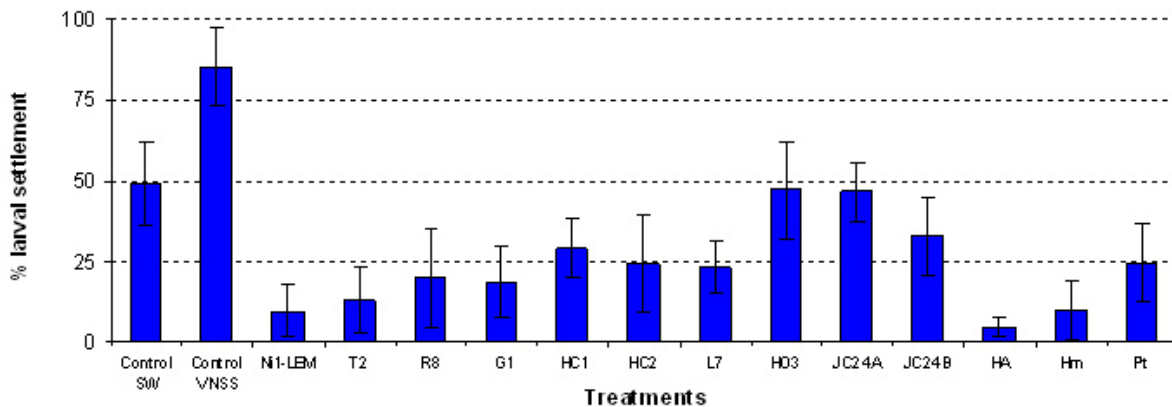

Figure 1. Effect on the settlement of *C. intestinalis* larvae exposed to different bacterial biofilms. Results represent settlement after 48 hrs. All results represent means of three replicate trials. Vertical bars = ± standard errors.

Table 1. Epibiotic bacterial strains isolated from different substrates.

Organism/Substrate	Code assigned to Bacteria Isolate	Number bacterial strains
Athyonidium chilensis	HO1, HO2, HO3, HC1, HC2, HA, AA1, AA3	8
Ciona intestinalis	T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, JC14, JC19A, CJ19B, CJ24A, CJ24B, CJ24C	16
Glosophora	G1, G2, G3, G4, G5	5
Lithothamnium	L1, L2, L3, L4, L5, L6, L7,L8, L9, L10	10
Perumytilus algosus	CH1, CH2, CH3, CH4, CH5	5
Rhodymenia	RHO1, RHO2, RHO3, RHO4, RHO5, RHO6, RHO7, R8, Ni1	9
Tetrapigus niger	TA1, TA2, TA3, TO1	4
Ulva lactuca	U1, U2, U3, U4, U5, U6, U7, U8,U9,U10	10
Polypropylene plates	C1, C2,C3, C4, C5, Ni1-LEM	6
Total bacterial strains isolated		**73**

fungal infections (Gil-Turnes and Fenical, 1992). Biofilms of the bacteria *Pseudoalteromonas tunicata*, isolated from the surface of a tunicate, showed antifouling activity against larvae of *Balanus amphitrite* and *C. intestinalis* (James et al. 1996; Holmström and Kjelleberg, 1999).

Ciona intestinalis (white sea squirt) is an important cosmopolitan fouling organism on both natural and artificial substrates (Connell, 2000; Mazouni et al. 2001). This tunicate produces serious operational difficulties in diverse aquaculture centres, for example in Scotland (Karayucel, 1997), S. Africa (Hecht and Heasman, 1999), and Chile (Uribe and Etchepare, 2002). In Chile, this problem is particularly costly in the culture of the scallop *Argopecten purpuratus*, where masses of tunicates weigh down lines and cage culture systems, compete with the scallops for food and oxygen, reduce the transport capacity of small workboats, and require considerable investment in labour and equipment for cleaning culture apparatus (FIP, 1996). Another tunicate, the "red sea squirt" *Pyura praeputialis*, is an introduced species in Chile which is becoming a dominant component in the rocky intertidal environment near Antofagasta (Castilla et al. 2002) and causes serious problems for scallop culture in this region.

The main objective of the present study was to search for indigenous marine bacteria occurring in biofilms which exerted inhibitory activity on the settlement of larvae of the tunicates common in the fouling of *A. purpuratus* culture systems. A long term goal of this work was to concentrate natural antifouling materials produced by bacteria in a way that they could be employed in fouling prevention without toxic side effects for the organisms in culture. The culture of *A. purpuratus* has become very important in Chile, making it the world's third largest exporter of scallops. Successful continuation and growth of this enterprise will depend, however, on successful development of measures for the control of its associated marine macrofouling problem.

MATERIALS AND METHODS

Larval culture

Ciona intestinalis. Adult *C. intestinalis* were obtained from the El Golfo Culture Center located at San Jorge Bay, Antofagasta, Chile (Lat 23º39'S) in spring 2004, and placed in a 500 L holding aquarium at the nearby Marine Science Faculty, University of Antofagasta. The tank received a constant flow of 10 μm filtered seawater at 18ºC and the ascidians were fed *ad libitum* with pure cultures of microalgae *Chaetoceros calcitrans* and *Ch. gracilis*. Mature individuals of over 60 mm in length were selected on the basis of the presence of spermatozoa and oocytes in the gonoducts as observed through the (transparent) body wall; these specimens were transferred to 2 L recipients containing 1 μL filtered seawater. Mature oocytes were
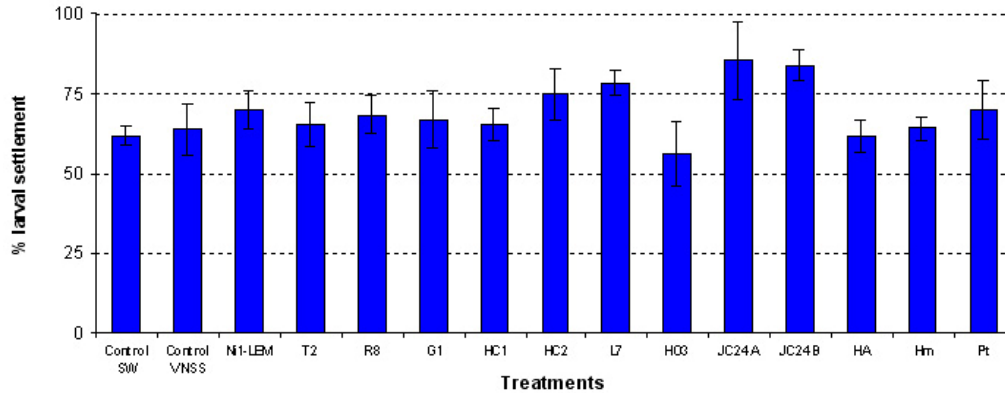
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Figure 2. Effect of different chloroform-killed bacterial strains from Figure 1 on settlement of *C. intestinalis* larvae. Results represent settlement after 48 hrs. All results represent means of three replicate trials. Vertical bars = ± standard errors.

collected by dissection of the distal oviduct next to the gonopore; oocytes were aspirated into a micropipette, depositing a total of about 300 oocytes into 15 ml of 0.2 µm filtered seawater in a Petri dish. Spermatozoa were collected from the sperm ducts of fresh specimens by the same method. Fertilization of the oocytes was carried out as aseptically as possible in a petri dish, allowing 30 min for the process. Success of fertilization step was checked using light microscopy at 40 X. The zygotes were gently rinsed with three changes of 0.2 µm filtered seawater and placed in a beaker with 500 mL 0.2 µm filtered seawater with aeration, and maintained at 18ºC until the larval stage was reached.

Pyura praeputialis. Adult specimens were collected at low tide from the rocky intertidal zone near Antofagasta (Lat 23º39'S) in spring 2004, and were returned to the laboratory and handled similarly to *C. intestinalis* (above). Artificial fertilization was carried out using the techniques previously described by Clarke et al. (1999).

Bacterial isolations

Natural and artificial substrates sampled for naturally occurring film-forming bacteria are listed in Table 1. These were rinsed copiously with sterile seawater to remove debris and weakly adhered microorganisms (Jiang et al. 1999). Samples of strongly adherent epibiotic bacteria were obtained using a bacteriological loop. Samples were spread on TCBS Agar (Oxoid Ltd.) and Zobell 2216 Marine Agar (Difco, Detroit, Michigan, USA). The TCBS agar plates were incubated at 20ºC for 48 hrs, and marine agar plates at the same temperature for one week. Once the colonies had developed, isolates were obtained by using a sterile loop and by spreading on TSA agar supplemented with 2% NaCl (TSA2) (Oxoid Ltd.). Various criteria were used in the selection of a broad variety colonies, including pigmentation, consistency, swarming behaviour, and others, which aided in recognizing them on the TSA agar (Table 1). Two bacterial strains previously known to inhibit

invertebrate larval settlement were included in the study as positive controls, including *Pseudoalteromonas tunicata* (Pt) provided by Dr. S. Kjelleberg and *Halomonas marina* (Hm) (Dobson and Franzmann, 1996) was obtained from the American Type Culture Collection (Manassas, Virginia, USA).

A preliminary characterization of the bacterial strains had inhibitory effects on the settlements of *C. intestinalis* larvae (Gram reaction and motility) was made using light microscopy. The biochemical tests catalase and O/F reaction with glucose (Gerhardt et al. 1994) were also carried out.

Preparation of bacterial films and their effects on *C . intestinalis* larval settlement

Biofilms were prepared from the 73 bacterial strains isolated, as well as from the two control species, using the methodology given by Maki et al. (2000). Overnight bacterial cultures grown in the medium VNSS, (Holmström et al. 1998) were centrifuged at 5520 x g for 15 min. The pellet was washed and resuspended in artificial seawater (ASW, "Sea Salt", Sigma Co.), and re-centrifuged. The pellet was then resuspended in 1/10 VNSS/ASW. The bacterial count in this suspension was determined using DAPI (4',6'-diamidino-2-phenyllindole) solution (ca. 1 µg mL^{-1}), followed by observation with a fluorescence microscope (Olympus BH-2) within 30 min (Kogure et al. 1998). Three mL of each bacterial suspension, at a concentration of 10^8 cells mL^{-1}, were deposited into 30 mm. diameter polystyrene dishes, which were then incubated at 20ºC for 48 hrs. The culture medium was then decanted and the plates were rinsed 3 times with ASW to eliminate cells which were not adhered to the substrate. Controls were prepared by the above steps, but omitting bacteria from the system. An additional set of trials included preparation of a parallel set of bacterial films which were then killed by a 30 min exposure to chloroform fumes.

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Table 2. Comparison of results from the present study with similar studies on other invertebrates and one algal species.

Test species	# bacterial strains isolated	% positive	effect tested	reference
C. intestinalis	73	20	settlement	present study
P. praeputialis	73	20	settlement	present study
C. intestinalis	40	12.5	settlement	Holmstrom et al. 1992
B. amphitrite	40	12.5	settlement	Holmstrom et al. 1992
B. amphitrite	16	64	settlement	Mary et al. 1993
Hydroides elegans	11	18	settlement	Dobretsov and Quian, 2004
Ulva (spores)	56	23	spore germination	Egan et al. 2001

Larvae settlement assays of *C. intestinalis* were done by introducing 15 larvae in a total of 3 ml seawater into all dishes containing living and killed bacterial biofilms, plus controls without bacterial films. The systems were incubated at 18ºC for 48 hrs, and then observed using an inverted microscope to determine the numbers of settled and non-settled tunicate larvae. The percentage settlement as then calculated for all systems. Three replicate trials were made in all the experiments.

Preparation of bacterial extracellular products (EP)

Extracellular fractions were prepared from bacterial strains Ni1-LEM and HA, and the positive controls Pt and Hm, all of which had been cultured to the stationary phase in Marine Minimal Medium (MMM) (Maki et al. 2000) with constant stirring at room temperature (18ºC). The cultures were centrifuged at 18600 x g for 15 min at 4ºC, and the supernatant was filtered twice to 0.2 μm and stored at -70ºC.

Inert matrix preparation for larvae settlement assays of *C. intestinalis* and *P. praeputialis*

The inert polymer Phytagel^{TM} (Sigma Chemical Company), was prepared by diluting 0.326 g of the product in 20 mL of distilled water with stirring, and then heated for 60 sec in a microwave oven, to reach 80ºC. At the same time, the extracellular products from the four bacterial strains obtained above were brought to 45-50ºC, and incorporated into the Phytagel. Testing as carried out using two different final dilutions (1:2 and 1:10) of the EP in the Phytagel. Controls were prepared with Phytagel only, Phytagel with sterile seawater, and Phytagel with MMM. TwentymL of the molten Phytagel were deposited in 30 mm polystyrene petri dishes and allowed to solidify at room temperature. Each experimental and control plates received three ml

aliquots of seawater containing 15 *C. intestinalis* larvae; and a parallel test was run using the same numbers of *P. praeputialis* larvae. Settlement of the larvae in each plate was determined after 48 hrs by direct observation in an inverted microscope.

EP heat treatment

In order to determine the effects of heat treatment on the bacterial EP, the extracts were treated at 80ºC in an incubator bath for 30 min, and allowed to cool to room temperature, filtered to 0.2 μm. The normal and heat treated EP were tested in parallel on their capacity to inhibit settlement of *C. intestinalis* and *P. praeputialis* larvae.

EP fractionation

The approximate molecular size bacterial antifouling compounds was determined using dialysis by fractionation from bacterial strains Nil-LEM and HA by dialysis against sterile distilled water for 12 hrs at 4ºC with benzoilated membranes (Sigma Co.) with a cut-off of 3,500 and 10,000 Da. The dialysates were filtered to 0.2 μm. and used in larval settlement tests. Controls included seawater and MMM.

Enzymatic treatment of the EP

An assay was carried out to determine if the EP factors inhibitory to tunicate settlement were proteic or peptidic in nature. For this the EP were separately treated at a final concentration of 200 μgmL^{-1} pronase E and carboxypeptidase G. The pronase E mixture was incubated at 37ºC for two h and the carboxypeptidase G at 30ºC for 3 hrs, both at pH 7.1. The treated PE were then heated to 80ºC for 20 min to inactivate all proteic and enzymatic activity. Controls for both treatments included seawater and MMM substituted for EP.
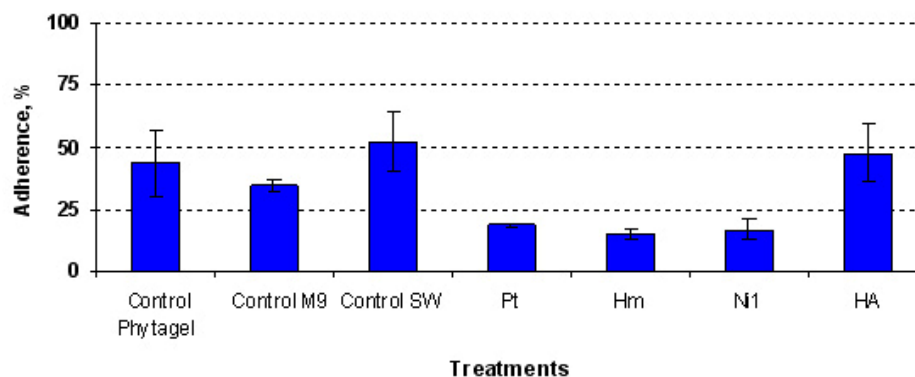
Figure 3. The effect of bacterial extracellular products (EP) incorporated into Phytagel™ on the settlement of *C. intestinalis* larvae after 48 hrs incubation. Results represent the means of three replicate assays. Vertical bars = ± standard errors.

Separation of EP fractions using high pressure liquid chromatrography (HPLC)

The supernatants from pre-stationary phase cultures of the bacterial strain Ni1-LEM were concentrated by vacuum evaporation at 70ºC (Rotavap Büchi) for 4 hrs. One-mL samples were then separated by HPLC on a C_{18} column with in a gradient of acetonitrile:water with 0.1% trifluoroacetic acid. Fractions were collected at 4, 5, 6, 7, 8, 9, 13, 17 and 18 min intervals. One-mL fractions were collected and concentrated by vacuum centrifugation in a speedvac (Centrivap console, Labconco), at 60ºC and were used in bioassays for antifouling activity using five larvae/ml of *C. intestinalis* and *P. praeputialis.* This assay was carried out in multi-well polystryrene plates, with 30 mm diameter wells, containing 3 mL seawater with 15 larvae per well. Controls were run, as previously, with seawater and MMM substituted for EP. The numbers of larvae settled were determined by observation with an inverted microscope after 48 hrs incubation as in other trials (above).

Statistical analyses

One-way analyses of variance (ANOVA, $\alpha = 0.05$) were used to evaluate the effects of both living and non-living biofilms, as well as the effects of the bacterial extracellular components, on the inhibition of *C. intestinalis* and *P. praeputialis* larvae settlement. The determination of the factors which were responsible for significant differences was carried out using a multiple comparison, test (Tukey-HSD).

RESULTS

Effect of the bacterial biofilm on *C. intestinalis*

A total of 73 film-forming bacterial strains were isolated (Table 1). The assays with living bacterial films showed that 20% of the strains had inhibitory effects on the settlement of *C. intestinalis* larvae; strong inhibition was indicated when 50% or more of the larvae present failed to settle in the plates. These bacterial strains isolated were gram-negative, fermentative, positive for motility and catalase.

Figure 1 shows the bacterial strains having the highest antifouling effects, as well as the positive (Hm, Pt) and negative controls. Significant differences were observed in averages of non-settled larvae between treatments and negative controls (ANOVA, $p < 0.05$).

The inhibitory ability of the biofilms was lost when they were tested in the non-living condition, as shown by Figure 2. No significant differences were found in the percentages of non-adhered larvae between (non-living) biofilmed surfaces and controls (ANOVA, $p < 0.05$).

Effect of immobilized PE on larvae of *C. intestinalis*

The extracellular products incorporated into the Phytagel which had inhibitory effect was strain Ni1-LEM (16.8%). Significant differences (ANOVA, $p < 0.05$) occurred between the results obtained with strains Pt, Hm (positive controls) and Ni1-LEM, and the negative controls (Figure 3).

Effect of bacterial EP on *C. intestinalis* and *P. praeputialis* larvae

The EP from bacterial strains Pt, Hm and Ni1-LEM did not lose their ability to inhibit settlement of larval *C. intestinalis* and *P. praeputialis* after dilution and/or denaturation (Figure 4 and Figure 5). Here, significant differences were detected between the treatments and the negative controls (ANOVA, $p < 0.05$). The bacterial strains Pt, Hm, and Ni1-LEM lost their antifoulimg activity against *C. intestinalis* after exposure to protease, although peptidase had no effect. A contrasting result was obtained with strain HA (Figure 4) which lost its antifouling activity after exposure to peptidase, but not with protease. This
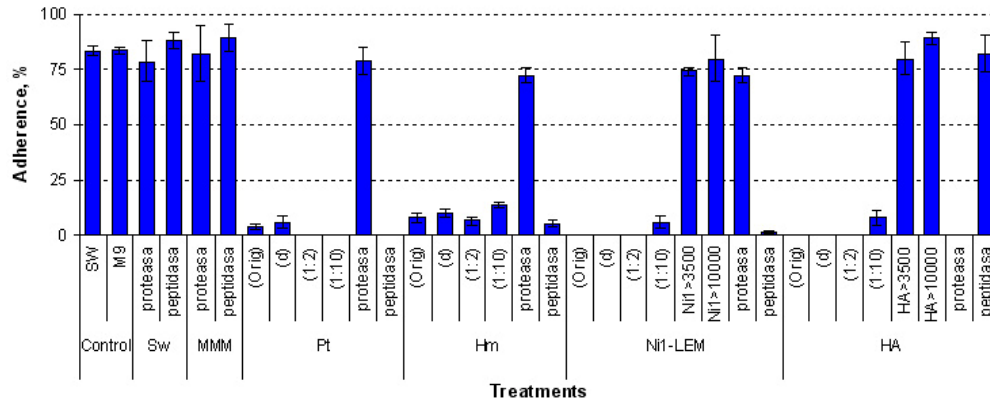
Figure 4. Effects of bacterial extracellular products (EP) on settlement of *C. intestinalis* larvae after a 48 hrs incubation. (Orig) = untreated EP; (d) = heat denatured EP; (1:2) and (1:10) = EP dilutions; Ni1 > 3,500 and Ni1 > 10,000 = dialyzed fractions of EP; protease and peptidase = EP enzymatic treatments. All values are the results of three replicate trials. Controls: SW = seawater; MMM = marine minimal medium; Pt and Hm = positive control bacterial strains. Vertical bars = ± standard errors.

could be indicate that EP of the strain HA are peptide origin.

Figure 5 shows that the EP of strains Pt, Hm, Ni1-LEM and HA lost their inhibitory effect against *P. praeputialis*, after treatment with proteolytic enzymes. Dialysis of the PE showed that substances inhibitory to the settlement of *C. intestinalis* and *P. praeputialis* larvae produced by strains Ni1-LEM and HA had molecular weights of less than 3,500 Da.

Effect of EP HPLC fractions on settlement of *C. intestinalis* and *P. praeputialis* larvae

Figure 6a shows the results with the EP fractions obtained by separation with an HPLC column on larvae settlement of the two ascidian species used in this study. Only the four- and five-minute column eluates of the PE from bacterial strain Ni1-LEM inhibited larval settlement of the two

ascidian species (Figure 6a). The remaining fractions produced no significant differences from the controls (ANOVA, p < 0.05).

DISCUSSION

The present results are in general agreement with results obtained by other authors working with various species as listed in Table 2.

The results of these studies and those of the present study showed that bacteria present in biofilms may have an important role in the development of trophic successions in benthic micro systems based on inhibitory ability of microfouling components of the intertidal zone. The presence of the inhibitory bacteria can influence the normal development and survival of some invertebrates and algae (Egan et al. 2001). The inhibitory ability of these biofilms is lost after killing with chloroform (Figure 2), suggesting
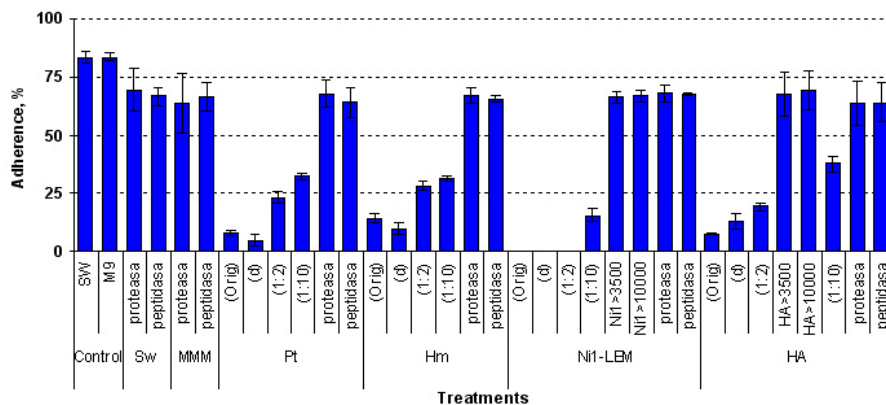

Figure 5. Effects of bacterial extracellular products (EP) on settlement of *P. praeputialis* larvae after a 48 hrs incubation. (Orig) = untreated EP; (d) = heat denatured EP; (1:2) and (1:10) = EP dilutions; Ni1 > 3,500 and Ni1 > 10,000 = dialyzed fractions of EP; protease and peptidase = EP enzymatic treatments. All values are the results of three replicate trials. Controls: SW = seawater; MMM = marine minimal medium; Pt and Hm = positive control bacterial strains. Vertical bars = ± standard errors.

that the production of inhibitory substances is an activity inherent to the metabolism of living cells. In preliminary test chloroform fumes, no affects the activity inhibitory (data unpublished).

Incorporation of bacterial inhibitory substances in a gel matrix is a model system representing a method for applying antifouling substances onto surfaces in need of antifouling protection in the sea. The antifouling compounds can be incorporated into the matrix at concentrations higher than those in the natural environment without altering the physical characteristics of the settlement surface (Henrikson and Pawlik, 1995). This technique is valuable for assaying the antifouling effects of secondary metabolites produced by marine organisms (Henrikson and Pawlik, 1998). Phytagel^TM was chosen for the present study due to its stability when compared with other potentially useful polymers. Henrikson and Pawlik (1995) have reviewed these properties related to their *in situ* studies with extracts from marine invertebrates. As noted above, the inhibitory ability of bacterial extracellular products was not inhibited by their inclusion in Phytagel, with one bacterial strain (HA) as an exception (Figure 3). The observed increases of effectiveness of the bacterial

extracellular products with higher concentration (Figure 4 and Figure 5) were similar to the results of Thiyagarajan et al. (1999), where low concentrations of extracts did not affect settlement of *B. meticulatus*, but high concentrations did.

Our results suggest that the bacterial inhibitory substances are thermostable, proteic or peptidic substance of less than 3500 Da in MW (strain Ni1-LEM); this agrees with Holmström and Kjelleberg (1999) and Egan et al. (2001) that low molecular weight peptides or proteins inhibited the settlement of bacteria, fungi, microalgae, algal spores, and invertebrates. In contrast, Dobretsov and Qian (2004) found that inhibitory substances isolated from bacteria from the soft coral *Dendronephthya sp* had a molecular weight of greater than 100 KDa.

The results obtained from the HPLC analyses suggested that the protein or peptide present in the active fraction had polar characteristics (in preliminary experiments it was test, data no show) (Figure 6), similarly to that reported by Egan et al. (2001), polar proteins and low molecular weight, which inhibited the germination of *Ulva lactuca* spores. Previously, Kawamata et al. (1994) reported that substances
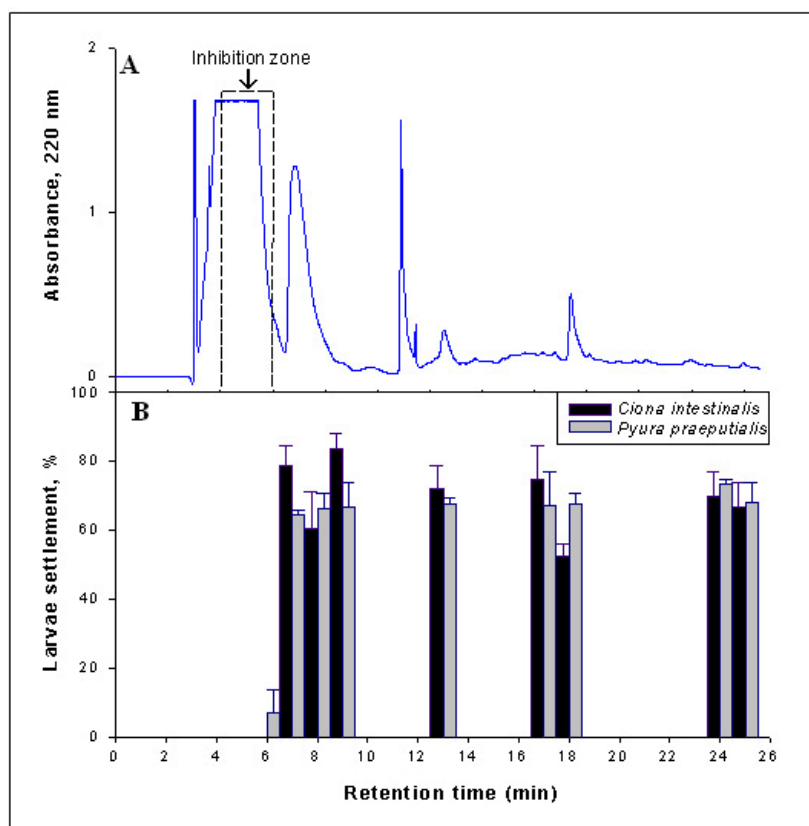

Figure 6. Larval settlement results obtained with strain Ni1-LEM EP fractions separated by HPLC. **(a) The chromatograph shows absorbance of the EP at 220 nm over time (min).** The broken line represents fractions showing anti-settlement activity.
(b) Effects of HPLC-separated PE fractions from bacterial strain Ni1-LEM on larvae settlement of *C. intestinalis* and *P. praeputialis* after a 48 hrs incubation. All results are means of three replicate trials. Vertical bars = ± standard errors.

from the soft coral *Dendronephthya sp* which inhibited the settlement of *Balanus amphitrite* larvae were water soluble and of low molecular weight. Although, we can not dismissed the presence of other compounds of other chemical nature present in the bacterial supernatants.

Indirect evidence, as well as newly emerging experimental evidence, suggests that many marine species have variable abilities for protecting themselves from overgrowth by natural competitors and neighbours within their communities. Protective substances may be produced directly by the organisms, or may be products from microbial symbionts or incidental microbial film-formers in the habitat. In any case, extracellular metabolites with antifouling activity are metabolic products representing an energy cost to the producers, which may be why in the few reports we have seen (Table 2) bacterial antifouling activity has been relatively low (only 20% of the strains were active in the present study). It is of pressing interest to find unique substances of bacterial or other origin which would be useful for incorporation into antifouling coatings of practical use. As is well known for existing chemical antifouling coatings such as those containing copper or TBT, they are designed to release considerable quantities of highly toxic substances to be effective over time. It is therefore essential to find naturally repellent products with a broad spectrum activity and capable of being made available in commercial quantities.

The present study is part of the beginning of an effort to find natural products which in the future will fulfil emerging needs for antifouling products which repel classically damaging invertebrates, while at the same time allowing the growth of desirable species in aquaculture.

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REFERENCES

ARMSTRONG, Evelyn; BOYD, Kenneth G.; PISACANE, Tony; PEPPIAT, C.J. and BURGESS, J. Grant. Marine microbial natural products in antifouling coatings. *Biofouling*, 2000, vol. 16, no. 2-4, p. 215-224.

BURGESS, J. Grant; BOYD, Kenneth G.; ARMSTRONG, Evelyn; JIANG, Zhong; YAN, Liming; BERGGREN, Matz; MAY, Ulrika; PISACANE, Tony; GRANMO, Ake and ADAMS, David R. The development of a marine natural product-based antifouling paint. *Biofouling*, 2003, vol. 19, no. 1, p. 197-205.

BOYD, K.G.; MEARNS-SPRAGG, A.; BRINDLEY, G.; HATZIDIMITROU, K.; RENNIE, R.; BREGU, M.;

HUBBLE, M.O. and BURGESS, J.G. Antifouling potential of epiphytic marine bacteria from the surfaces of algae. In: LE GAL, Y. and MULLER-FEUGA, Arnaud eds. *Marine Microorganisms for Industry, Microorganismes marins pour l'industrie*. Editions IFREMER, Plouzané, France, 1998, p. 128-136.

BOYD, Kenneth G.; ADAMS, David R. and BURGESS, J. Grant. Antibacterial and repellent activity of marine bacteria associated with algal surfaces. *Biofouling*, 1999, vol. 14, no. 3, p. 227-236.

BOYD, Kenneth G.; MEARNS-SPRAGG, A. and BURGESS, J. Grant. Screening of marine bacteria for the production of microbial repellents using a spectrophotometric chemotaxis assay. *Marine Biotechnology*, July 1999, vol. 1, no. 4, p. 359-363.

CASTILLA, J.C.; COLLINS, A.C.; MEYER, C.P.; GUIÑEZ, R. and LINDBERG, D.R. and LINDBERG, D. Recent introduction of the dominant tunicate, *Pyura praeputialis* (Urochordata, Pyuridae) to Antofagasta, Chile. *Molecular Ecology*, August 2002, vol. 11, no. 8, p. 1579-1584.

CLARE, A.S. Marine natural product antifoulants: status and potential. *Biofouling*, 1996, vol. 9, no. 3, p. 211-229.

CLARKE, M.; ORTIZ, V. and CASTILLA, J.C. Does early development of the Chilean tunicate *Pyura praeputialis* (Heller, 1878) explain the restricted distribution of this species? *Bulletin of Marine Science*, 1999, vol. 65, p. 745-754.

CONNELL, Sean D. Floating pontoons create novel habitats for subtidal epibiota. *Journal of Experimental Marine Biology and Ecology*, May 2000, vol. 247, no. 2, p. 183-194.

DOBRETSOV, Sergey V. and QIAN, Pei-Yuan. Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro-and macrofouling. *Biofouling*, 2002, vol. 18, no. 3, p. 217-228.

DOBRETSOV, Sergev V. and QIAN, Pei-Yuan. The role of epibiotic bacteria from the surface of the soft coral *Dendronephtya* sp. in the inhibition of larval settlement. *Journal of Experimental Marine Biology and Ecology*, February 2004, vol. 299, no. 1, p. 35-50.

DOBSON, S.J. and FRANZMANN, P.D. Unification of the genera *Deleya* (Baumann et al. 1983), *Halomonas* (Vreeland et al. 1980), and *Halovibrio* (Fendrich, 1988) and the species *Paracoccus halodenitrificans* (Robinson and Gibbson, 1952) into a single genus; *Halomonas*, and placement of the genus *Zymobacter* in the family *Halomonadaceae*. *International Journal of Systematic Bacteriology*, April 1996, vol. 46, no. 2, p. 550-558.

EGAN, Suhelen; JAMES, Sally; HOLMSTRÖM, Carola and KJELLEBERG, Staffan. Inhibition of algal spore germination by the marine bacterium *Pseudoalteromonas tunicata*. *FEMS Microbiology Ecology*, March 2001, vol. 35, no. 1, p. 67-73.

FIP (Fisheries Research Fund). Technical Report FIP-IT/96-55. Investigación de variables que influyen en el desarrollo del piure blanco en cultivos de ostión. Universidad Católica del Norte, Coquimbo. Chile, 1996, p. 110.

FISHER, W.S.; OLIVER, L.M.; WALKER, W.W.; MANNING, C.S. and LYTLE, T.F. Decreased resistance of eastern oysters (*Crassostrea virginica*) to a protozoan pathogen (*Perkinsus marinus*) after sublethal exposure to tributyltin oxide. *Marine Environmental Research*, March 1999, vol. 47, no. 2, p. 185-201.

GERHARDT, P.; MURRAY, R.G.E.; WOOD, W.A. and KRIEG, N.R. *Methods for General and Molecular Bacteriology*. American Society for Microbiology, Washington, 1994, 791 p. ISBN 1-55-581048-9.

GIL-TURNES, M.S. and FENICAL, W. Embryos of *Homarus americanus* are protected by epibiotic bacteria. *The Biological Bulletin*, February 1992, vol. 182, no. 1, p. 105-108.

GRINWIS, G.C.M.; BOONSTRA, A.; VAN DEN BRANDHOF, E.J.; DORMANS, J.A.M.A.; ENGELSMA, M.; KUIPER, R.V.; VAN LOVEREN, H.; WESTER, P.W.; VAAL, M.A.; VETHAAK, A.D. and VOS, J.G. Short-term toxicity of bis (tris-*n*-butylin) oxide in flounder (*Platichthys flesus*): Pathology and immune function. *Aquatic Toxicology*, May 1998, vol. 42, no. 1, p. 15-36.

HARDER, Tilmann; LAU, Stanley C.K.; DOBRETSOV, Sergey; FANG, Tsz K. and QIAN, Pei-Yuan. A distinctive epibiotic bacterial community on the soft coral *Dendronephthya* sp. and antibacterial activity of coral tissue extracts suggest a chemical mechanism against bacterial epibiosis. *FEMS Microbiology Ecology*, April 2003, vol. 43, no. 3, p. 337-347.

HECHT, Thomas and HEASMAN, Keavin. The culture of *Mytilus galloprovincialis* in South Africa and the carrying capacity of mussel farming lfz Saldanha Bay. *World Aquaculture Society*, 1999, vol. 30, no. 4, p. 50-55.

HENRIKSON, Alicia A. and PAWLIK, Joseph R. A new antifouling assay method: results from field experiments using extracts of four marine organisms. *Journal of Experimental Marine Biology and Ecology*, December 1995, vol. 194, no. 2, p. 157-165.

HENRIKSON, Alicia A. and PAWLIK, Joseph R. Seasonal variation in biofouling of gels containing extracts of marine organisms. *Biofouling*, 1998, vol. 12, no. 1-3, p. 245-255.

HENTSCHEL, Ute; SCHMID, Michael; WAGNER, Michael; FIESELER, Lars; GERNERT, Christine and HACKER, Jörg. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiology Ecology*, May 2001, vol. 35, no. 3, p. 305-312.

HOLMSTRÖM, Carola; RITTSCHOF, Dan and KJELLEBERG, Staffan. Inhibition of settlement by Larvae of *Balanus amphitrite* and *Ciona intestinalis* by a surface-colonizing marine bacterium. *Applied and Environmental Microbiology*, July 1992, vol. 58, no. 7, p. 2111-2115.

HOLMSTRÖM, Carola; JAMES, S.; NEILA, B.; WHITE, D. and KJELLEBERG, Staffan. *Pseudoalteromona*s tunicata sp. nov., a bacterium that produces antifouling agents. *International Journal of Systematic Bacteriology*, October 1998, vol. 48, no. 4, p. 1205-1212.

HOLMSTRÖM, Carola and KJELLEBERG, Staffan. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiology Ecology*, December 1999, vol. 30, no. 4, p. 285-293.

JAMES, Sally G.; HOLMSTRÖM, Carola and KJELLEBERG, Staffan. Purification and characterization of a novel antibacterial protein from marine bacterium D2. *Applied and Environmental Microbiology*, August 1996, vol. 62, no. 8, p. 2783-2788.

JIANG, Zhi-Dong; JENSEN, Paul R. and FENICAL, William. Lobophorins A and B, new antiinflammatory macrolides produced by a tropical marine bacterium. *Bioorganic and Medicinal Chemistry Letters*, July 1999, vol. 9, no. 14, p. 2003-2006.

KARAYUCEL, S. Mussel culture in Scotland. *World Aquaculture Society*, 1997, vol. 28, p. 4-10.

KAWAMATA, M.; KON-YA, K. and MIKI, W. Trigonelline, an antifouling substance isolated from an octocoral *Dendronephthya* sp. *Fisheries Science*, 1994, vol. 60, p. 485-486.

KOGURE, Kazuhiro; IKEMOTO, Eiko and MORISAKI, Hisao. Attachment of *Vibrio alginolyticus* to glass surfaces is dependent on swimming speed. *The Journal of Bacteriology*, February 1998, vol. 180, no. 4, p. 932-937.

MAKI, J.S.; DING, L.; STOKES, J.; KAVOURAS, J.H. and RITTSCHOF, D. Substratum/Bacterial interaction and larval attachment: Films and exopolysaccharides of *Halomonas marina* (ATCC 25374) and their effect on barnacle cyprid larvae, *Balanus amphitrite* Darwin. *Biofouling*, 2000, vol. 16, no. 2-4, p. 159-170.

MARY, Avelin; MARY, Vitalina; RITTSCHOF, D. and NAGABHUSHANAM, R. Bacterial-barnacle interaction:

Potential of using juncellins and antibiotics to alter structure of bacterial communities. *Journal of Chemical Ecology*, October 1993, vol. 19, no. 10, p. 2155-2167.

MAZOUNI, N.; GAERTNER, J.-C. and DESLOU-PAOLI, J.-M. Composition of biofouling communities on suspended oyster cultures: an in situ study of their interactions with the water column. *Marine Ecology - Progress Series*, April 2001, vol. 214, p. 93-102.

PEPPIATT, C.J.; ARMSTRONG, E.; PISACANE, T. and BURGUESS, J.G. Antibacterial activity of resin based coatings containing marine microbial extracts. *Biofouling*, 2000, vol. 16, no. 2-4, p. 225-234.

THIYAGARAJAN, V.; MURTHY, S.P.; NANCHARAIAH, Y.V.; VENUGOPALAN, V.P.; NAIR, K.V.K. and SUBRAMONIAM, T. Influence of biofilms on the larval settlement of *Balanus reticulstus* Utinomi (Cirripedia: Crustacea). *Biofilm*, 1999, vol. 4, paper (BF99001). Available from Internet: http://www.bioline.org.br/abstract?id=bf99001&lang=en.

URIBE, E. and ETCHEPARE, I. Effects of biofouling by *Ciona intestinalis* on suspended culture of *Argopecten purpuratus* in Bahía Inglesa, Chile. *Bulletin of the Aquaculture Association of Canada*, 2002, vol. 102, p. 93-95.

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