Chemical control of bacterial epibiosis on ascidians

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ABSTRACT: Two co-occurring ascidians of the Pacific subtidal, *Polyclinum planum* and *Cystodytes lobatus*, were found to exhibit remarkably different numbers of surface bacteria. On average, epibacterial densities on *P. planum* were 140 times greater than those on *C. lobatus* as measured by plate-counting methods. Organic extracts of both ascidian species were tested for antimicrobial activities and effects on bacterial settlement. Bacterial settlement was measured using a new bioassay described in this paper. The results of the settlement bioassay clearly demonstrate that extracts of the little-colonized *C. lobatus* deter bacterial settlement while extracts of the heavily colonized *P. planum* induce settlement relative to the control. In addition to reducing bacterial settlement, extracts of *C. lobatus* colonies exhibited varying, but generally low, levels of antimicrobial activity against, on average, one-half of the 36 strains of marine bacteria tested. On an interspecific level, including 11 ascidian species screened in a pilot study, fouling-deterring activities were correlated with epibacterial abundances while antimicrobial activity was not. It is concluded that the chemical control of bacterial settlement, possibly complemented by antimicrobial toxicity, provides an accurate model to explain the dramatically different bacterial abundance on the surfaces of the ascidian species studied. This investigation presents evidence that non-toxic metabolites influence bacterial settlement and, in this way, may function to regulate bacterial epibiosis on the surfaces of some marine invertebrates.

KEY WORDS: Ascidiacea · Bacterial attachment · Epibiosis · Chemotaxis · Settlement · Antimicrobial activity · Antisettlement activity · Chemical defense · Antifouling

INTRODUCTION

The discovery that bacteria rapidly colonize inanimate surfaces submerged in the sea was made more than 5 decades ago (Zobell & Allen 1935). Subsequent to this observation, the details of the colonization process, and their ecological significance, have been thoroughly described (Corpe 1970, Marshall et al. 1971, Costerton et al. 1978, Wardell et al. 1983, Mitchell & Kirchman 1984, Lappin-Scott & Costerton 1989). This process generally comprises 3 main phases: approach to a surface, reversible followed by irreversible attachment to that surface, and spreading by reproduction.

Bacteria, however, do not colonize all submerged surfaces in a uniform manner. Many factors, including the physical and chemical characteristics of the surface, influence the colonization process (Fletcher & Marshall 1982). In the case of living marine surfaces, the colonization process can additionally be affected by organic metabolites produced by the host organism. These metabolites may affect bacteria in a number of ways, ranging from the induction of a chemotactic response (Bell & Mitchell 1972), to the inhibition of bacterial growth or cell death (Sieburth & Conover 1965). The degree to which marine bacteria respond to plant and invertebrate metabolites could have a profound effect upon the distributions of bacteria on living surfaces; yet, to date, these responses remain poorly documented.

It can be generalized that living marine surfaces do not experience the relatively regular pattern of biofilm

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formation, and subsequent development of a fouling community, typically observed on inanimate surfaces (Burkholder 1973, Sieburth 1979). The bacterial populations associated with living surfaces, which are an important component in the development of a fouling community (Mitchell & Kirchman 1984), appear to be highly variable. This variability includes epibiotic bacterial densities covering more than 6 orders of magnitude, ranging from organisms with all but sterile surfaces (Droop & Elson 1966, Garland et al. 1982, Wahl & Lafargue 1990) to others that exhibit extremely high epibacterial densities (e.g. Sieburth 1975, Novak 1984). Qualitatively, living marine surfaces may harbor specialized, low-diversity bacterial communities (Gil-Turnes et al. 1989, Gil-Turnes & Fenical 1992), or communities that in other ways are distinct from that of the surrounding seawater and neighboring microhabitats (Lewis et al. 1985, Santavy & Colwell 1990, Johnson et al. 1991). Based upon the dramatic variability in bacterial populations associated with living surfaces, it can be proposed that certain surface characteristics of plants and animals play a role in the control of epibacterial distributions.

The apparent regulation of bacterial populations on living marine surfaces can be attributed, at least in part, to the enhancement or inhibition of the microbial colonization process (Sieburth 1975, 1979). Bacterial colonization can be reduced by one, or any combination of, ecological, mechanical, physical, and chemical factors associated with the surface biology of the host. These factors include grazing (Caron 1987), sloughing (Barthel & Wolfrath 1989), surface tension and energy (Linskens 1963, Becker & Wahl 1991), wettability (Dexter et al. 1975), pH (Sieburth 1968, Baker & Orr 1986) and secondary metabolite production (Harrison 1982, Wahl & Banaigs 1991). These surface-associated properties may represent defensive adaptations that enhance host survival by providing a mechanism to control epibiont populations.

The role of secondary metabolites as a chemical defense against epibiosis has been discussed (Bakus et al. 1986, Davis et al. 1989, Paul 1992). Studies in this area have largely addressed the effects of secondary metabolites upon invertebrate epibionts. These effects have been demonstrated most conclusively in the case of chemically mediated deterrence of larval settlement (see review by Pawlik 1992). There is also evidence that invertebrate secondary metabolites, tested at or below natural concentrations, can inhibit the growth of fouling diatoms (Targett et al. 1983, Bandurraga & Fenical 1985). The observation of an inverse correlation between macro-fouling and antimicrobial activity of a (potential) invertebrate host has been proposed as evidence that secondary metabolites are involved in the control of epibiosis (e.g. Al-Ogily & Knight-Jones

1977, McCaffrey & Endean 1985, Thompson et al. 1985, Davis et al. 1989, Uriz et al. 1991). However, the degree to which antimicrobial activities translate to *in situ* effects upon potential surface-colonizing organisms, especially with regard to microfouling, has not been determined.

The role of plant and invertebrate secondary metabolites in the control of bacterial epibiosis remains largely undocumented. It has been shown that phenolic substances produced by marine algae may correlate inversely with epibacterial distributions (Conover & Sieburth 1964, Sieburth & Conover 1965, Sieburth 1968, Cundell et al. 1977). However, this subject has not been thoroughly addressed, and, where reports exist, the extent to which antimicrobial secondary metabolite production correlates with reduced numbers of surface bacteria has been variable (Walls et al. 1993). Difficulties in correlating extract antimicrobial activity with effects upon epibacterial populations may be due to the absence of an ecologically relevant method to assess the chemical modulation of the bacterial colonization process.

In contrast to standard antimicrobial tests, measurements of bacterial attachment to a surface reflect multiple aspects of the colonization process, including chemotactic responses and factors affecting the firm attachment of cells to a substratum. Considering that bacteria respond tactically to a variety of metabolites (Chet & Mitchell 1976), including attraction to algal extracellular products (Bell & Mitchell 1972) and repulsion from tannic acid (Chet et al. 1975), the induction of a negative chemotactic response may be an important mechanism in the function of an effective chemical defense against microfouling. This response, on the other hand, may be overlooked in assessments of antimicrobial activity, as bacterial chemotactic responses can be observed at non-toxic chemical concentrations (Chet et al. 1975). Investigating the chemical control of bacterial attachment offers an informative method by which to assess the ecological role of secondary metabolites in regulating bacterial distributions and ultimately in the development of surface-associated microbial communities.

Using a recently developed bioassay, we examined the extracts of 13 ascidian species, collected off La Jolla, California, USA, for their ability to affect bacterial attachment to an agar surface. We observed a significant inverse correlation between epibacterial abundance and the ability of an extract to deter bacterial settlement. Conversely, no correlation was apparent between antimicrobial activity and epibacterial abundance. These results led us to more thoroughly investigate the surface bacterial communities of 2 ascidian species, *Cystodytes lobatus* and *Polyclinum planum*, and the nature of the relationship between

these communities and the chemical control of bacterial epibiosis. We propose that the results of the settlement bioassay, as described here, offer a method of assessing the role of host metabolites in the control of epibacterial distributions. Our studies were designed to determine: (1) if bacterial chemotaxis and attachment to agar surfaces are affected by ascidian extracts and, thereby, whether settlement deterrence can be considered a chemical defense against bacterial attachment, and (2) whether there is a correlation between surface bacterial abundance and settlement deterrence or antimicrobial activity.

METHODS

Sample collection. *Polyclinum planum* and *Cystodytes lobatus* were collected at Bureau of California Fisheries (BCF) Reef, La Jolla, California, between 11 and 18 August 1992. Two additional, independent *C. lobatus* collections (5 to 10 colonies each) were made on 22 and 23 August 1992 and used for bioassay-guided chemical evaluation. BCF Reef is a small, rocky patch reef, ca 10 m in diameter, located at a depth of 22 m, 1.5 km off the coast and 3 km north of the Scripps Institution of Oceanography pier. Scuba divers collected 24 *C. lobatus* and 12 *P. planum* colonies over the entire reef area. Each sample consisted of 1 distinct colony. Samples were placed in sterile plastic bags underwater and brought to the lab for immediate processing.

Surface-associated bacterial abundance, characterization and isolation. Prior to bacterial isolation, the entire surface of each sample was rinsed for 10 s with a stream of sterile seawater (water pressure equivalent to a 10 cm water column). Small portions of 8 specimens (4 of each species) were prepared for scanning electron microscopy (SEM) by fixation in 2.5% gluteraldehyde in calcium- and magnesium-free artificial seawater. This was followed by dehydration in an alcohol series, critical point drying, and platinum-gold coating. Specimen surfaces were examined and photographed using a Cambridge 360 SEM.

To examine their epibacterial communities, all 34 colonies were processed individually in the following manner: For *Polyclinum planum*, a sterile plastic film with a 0.5 cm^2 hole was placed on the specimen surface and the area within this hole was swabbed with a sterile cotton-tipped applicator. The applicator tip was placed in a tube containing 4 ml sterile seawater. This tube was vigorously vortex-mixed yielding dilution 1, which was serially diluted 1:10 with sterile seawater yielding dilutions 2 to 4. Fifty µl of dilutions 1 to 4 were inoculated onto the surface of agar plates (medium B1: 2.5 g peptone, 1.5 g yeast extract, 1.5 ml glycerol, 17 g agar, 750 ml filtered seawater, 250 ml deionized water)

and spread with a sterile glass rod and plate spinner. This procedure was repeated in duplicate for each colony by swabbing separate surface areas. The procedure for *Cystodytes lobatus* was identical to that for *P. planum*, but due to a lower bacterial abundance, the area swabbed was increased to 2.5 cm^2 and only dilutions 1 to 3 were plated.

Bacterial colonies were counted on B1 plates after 5, 10 and 15 d of room temperature incubation (20 to 25°C). The average number of colony-forming units for each sample was calculated from the dilution 1 plates for *Cystodytes lobatus* and either the dilution 1 or dilution 2 plates for *Polyclinum planum*. These counts were converted to bacteria per cm^2 of sample surface. This enumeration technique is known to underestimate the actual number of viable bacteria (Kogure 1979); however, the method is well suited for the comparison of *culturable* bacterial populations. Thus, our use of the term 'epibacterial abundance' as estimated from plate counts should be viewed with this restriction in mind.

For each sample, 1 plate with 2 to 20 well-isolated bacterial colonies was selected, and all isolates on these plates were categorized according to the following 11 criteria: Gram reaction by the KOH method (Buck 1982), bacterial colony size, pigmentation, surface (smooth or wrinkled), brilliance (shiny or dull), border (irregular or entire), and the ability to spread on an agar surface. Cells were observed using a phase contrast Olympus BHT microscope with 100× oil immersion objectives and 10× oculars (1000× magnification), and descriptions of cell shape, size, motility, and spore formation were recorded. Based on bacterial colony and cell characteristics, 1 to 3 of the most common bacterial types from each sample were isolated into pure culture, as judged by uniform colony morphology, by repeated transfer on B1 plates. Isolated strains were frozen at −80°C in liquid B1 with 10% glycerol added as a cryoprotectant.

Extraction procedures. Ascidian colonies, following bacterial isolation, were soaked individually in 200 ml of 70% methanol/30% ethyl acetate for 1 h. The specimens were not macerated to emphasize extraction of organic materials located in surface tissues. Following extraction, the specimens were removed and the resulting organic extracts dried by evaporation under reduced pressure. The extract residues were re-suspended in 20 ml of 100% methanol and these solutions were transferred to new vials to remove salt precipitates. The methanol soluble extracts were dried, weighed, and re-solubilized in methanol at a concentration of 10 mg ml^{-1}. This procedure was followed for all 24 *Cystodytes lobatus* and 12 *Polyclinum planum* colonies, and the resulting extracts were used in the antimicrobial and settlement bioassays.

Chemical separation. Collections of *Cystodytes lobatus* intended specifically for chemical evaluation were treated as independent replicates. These specimens were kept frozen prior to extraction at which time the outer surface layers of the ascidian colonies (ca 1 to 2 mm) were removed and extracted with 70 % methanol/30 % ethyl acetate. The resulting extracts were partitioned between hexane and methanol. The methanolic portion was further partitioned between butanol and water. The butanol soluble portions were fractionated by silica vacuum liquid chromatography (VLC). The column was gradient eluted with solvents of increasing polarity (from 100 % isooctane to 100 % ethyl acetate, then from 100 % methylene chloride to 100 % methanol). This process yielded 14 fractions. All fractionation steps were guided by settlement bioassays with the bacterial strain designated PP1. Final separation of the settlement inhibiting fraction was achieved with normal phase silica HPLC using 10 % methanol/chloroform.

Settlement bioassay. The settlement bioassay was designed to determine the effects of organic extracts, and pure metabolites, on bacterial attachment to an agar matrix. This assay is not restricted to the use of ascidian extracts and can detect substances that interfere with any number of behavioral or physiological processes associated with the attachment of bacteria to a surface. In this bioassay, extracts are added to molten agar at concentrations near or below those estimated to occur in the species. Incorporating the extract into an agar matrix ensures that, regardless of the extract tested, the bacteria encounter the same physically and topographically homogeneous surface. As a result, the effects observed on bacterial attachment to the treated vs control surfaces should represent chemical modulation of the attachment process.

Preparation of settlement slides (Fig. 1): In a 9 ml glass vial, 10 mg of dried extract were mixed with 100 µl of molten (60 °C) 2 % Nobel Agar (10 % wt/vol). Four replicate 20 µl drops of this solution were pipetted

AGAR DROPS WITH EXTRACT OR SOLVENT ALONE

AGAR OVERLAY

MICROSCOPE SLIDE

Fig. 1. Diagram of the agar-coated slide used in the settlement bioassay

onto separate microscope slides (the use of histological slides with enhanced tissue adhesion properties improved agar adherence). One control (agar plus solvent) and up to 3 different extracts were placed on each slide. The locations of the drops were marked with a diamond knife on the back of the slide. The slides were stored in a water-saturated atmosphere for 0.5 h to allow diffusion of the extract throughout the agar drop. An agar overlay was applied by dipping the slide into a molten 1 % Nobel agar solution (50 °C), wiping clean the underside of the slide and leaving it to solidify in a horizontal position. This process was repeated until the drops were completely covered and the overlay formed a perfectly smooth surface. The slides were again stored in a water-saturated atmosphere, this time for 1 h, to allow the extract to diffuse into the agar overlay. The final concentration of extract in the agar was estimated to be ca 5 % (wt/vol), which is slightly less than the concentrations calculated from the extraction of the surface layers of the ascidian (ca 7 % wt/vol).

Bacterial cultures: It is important that the bacteria used for the settlement bioassay meet a number of criteria. These criteria include motility and the ability to settle quickly on an agar surface. One bacterial strain (PP1), chosen for its ability to meet these criteria, was tested against all *Polyclinum planum* and *Cystodytes lobatus* extracts. Because antimicrobial tests revealed that growth of the strain PP1 was inhibited by most *C. lobatus* extracts, a second strain (C21-II), which was antimicrobially insensitive to all *C. lobatus* extracts, was also used in the bioassay. Strains PP1 and CL21-II were isolated as epibionts of *P. planum* and *C. lobatus*, respectively, and, therefore, represent bacteria that come in contact with, and are capable of attaching to, the surfaces of these ascidians.

Prior to performing settlement bioassays, strains PP1 and/or C21-II were grown overnight on a shaker (250 rpm) in 100 ml of low nutrient medium (0.5 g peptone, 0.3 g yeast extract, 0.6 ml 50 % glycerol, 250 ml distilled water, 750 ml seawater). After 18 h, this suspension was diluted 4 to 8 times with sterile seawater, depending upon cell density, and transferred to a sterile, 250 ml microscope-slide staining dish (Wheaton Scientific, Millville, NJ, USA, catalog no. 900200).

Settlement bioassays: The treated slides were positioned vertically and equidistantly in the microscope-slide holder (part of the staining dish assembly). The colonization process was initiated by placing the slides and holder into the staining dish containing the bacterial suspension. The experimental vessel was shaken at 60 rpm to ensure a homogeneous distribution of the bacteria. Slides coated with extract-free agar were used to monitor the progress of colonization during the
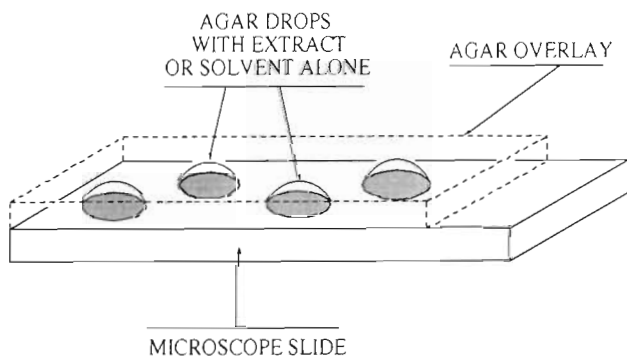
experiment. These slides were also placed in the staining dish and the density of settled bacteria was monitored every 5 min. After 10 to 15 min (when cells on the extract-free, agar-coated slides had reached the desired density of 10 to 100 cells field^{-1}), the slide holder was taken out of the bacterial suspension and the slides were rinsed cautiously with distilled water to prevent further bacterial attachment. This was followed by a more vigorous washing: all of the extracts and controls were rinsed with distilled water for 10 s from a distance of 1 cm with a water jet pressure of 10 cm. This procedure was intended to remove loosely attached cells and was found to reduce the variability between replicates.

It is of central importance that the settlement bioassay be of short duration. We observed that the ability of an extract to enhance or reduce settlement after a 15 min exposure could be lost after 30 min or longer. We ascribe this loss of activity to leaching of the extract from the agar matrix. The time necessary for an adequate number of cells to settle can be reduced by increasing the cell concentration. As settling characteristics of a given strain may vary, it is crucial to monitor the colonization process by examining the extract-free, agar-coated control slides every 5 min.

Bacterial counting: Bacteria attached to the agar surface were counted by epifluorescence microscopy after DAPI (4, 6-diamidino-2-phenylindole) staining. The bacteria were stained as follows: 5 µl of DAPI solution (20 µg ml^{-1}) was applied onto round coverslips (10 mm diameter); these were inverted and positioned onto the still wet agar surface over each extract and control. The DAPI was allowed to stain for at least 0.5 h before counting. It was confirmed by phase contrast microscopy that all cells were stained. At 1000× magnification, 10 fields were counted per replicate, and a minimum of 2 replicates were assessed per extract and colonizing strain. As colonizing rates were typically different for treatments and control on the same slide, field sizes had to be chosen so that between 10 and 100 cells could be counted per field. Four different counting fields were used: 64, 640, 1280, and 6400 µm^2. For the assessment of any given test spot, only 1 field size was used. The effect of an extract on bacterial attachment was expressed as number of cells on the extract-treated area divided by number of cells on the control area of the same slide (T/C). Small quotients thus stand for a strong reduction in bacterial settlement. The average factor (X) by which extracts of *Polyclinum planum* and *Cystodytes lobatus* affected bacterial attachment was calculated using the geometric mean ($\log X = 1/n \Sigma \log x$). In this way, activities of equal magnitude are weighed equally.

Antimicrobial bioassay. Extracts of *Polyclinum planum* and *Cystodytes lobatus* were tested for anti-microbial activity against a test panel of 40 terrestrial and marine microorganisms. The terrestrial strains included *Bacillus subtilis, Candida albicans,* and *Escherichia coli.* The marine bacteria included *Vibrio parahaemolyticus* (ATCC27969) and 36 strains (15 Gram positive, 21 Gram negative) isolated from the surfaces of *C. lobatus* (12 strains) and *P. planum* (24 strains). All extracts were tested using a standard paper disc diffusion assay. In this assay, 25 µl of extract at a concentration of 10 mg ml^{-1} were added to a paper disc (6 mm diameter). The solvent was allowed to evaporate and the disc was applied to the surface of a plate that had been inoculated with one of the test strains. Antimicrobial activity was measured as the area of no growth minus the area of the disc. Extracts responsible for no-growth areas ≥16 mm^2 (inhibition zone diameter ≥7.5 mm) were considered to possess antimicrobial activity.

Pilot study. In a previous survey (November 1991 through March 1992), we screened 13 ascidian species (including *Polyclinum planum, Cystodytes lobatus, Aplidium californicum* and *A. propinquum, Archidistoma psammion, Distaplia occidentalis, Metandrocarpa dura, Ritterella aequalisiphonis* and *R. pulchra, Synoicum parfustis,* 2 *Trididemnum* species and an unidentified white didemnid) for surface bacterial abundance, extract antimicrobial activity, and the effects of extracts on bacterial settlement. The animals were collected at 6 different localities near San Diego, California. The techniques used were essentially the same as described above, with the following exceptions: (1) Conspecific ascidian colonies (2 to 20) were extracted together ('pooled'). (2) The antibiotic tests were conducted against *Bacillus subtilis, Escherichia coli* and 11 bacterial strains isolated from the ascidian surfaces (5 Gram positive, 6 Gram negative). (3) The settlement tests were performed by adding 5 µg of extract (in solvent) or solvent alone (control) directly onto each of 4 marked locations on a microscope slide. After drying for 1 h at 30 °C, the settlement bioassay, as described above, was performed. The application of the extracts directly onto the glass slide (and not within an agar matrix) has the disadvantages that the extracts appeared to be less evenly distributed, the concentration of extract cannot be calculated, physical properties of the extract (e.g. hydrophobicity) may contribute to settlement effects, and water-soluble materials may be lost during the immersion phase. Conversely, this method requires much smaller quantities of extract (5 µg vs 2.5 mg), and was beneficial for the identification of settlement-inhibiting fractions during chemical isolation, when the quantity of material was often limited.

Some results of the pilot study are presented in this study. In this context we want to point out one weak-

ness of this preliminary screening: activities detected in pooled extracts may — theoretically — be caused by 1 active ascidian colony among several inactive conspecifics.

Data analysis. Throughout this investigation (i.e. sample collection, bacterial isolation, chemical extraction, etc.), individual colonies of *Polyclinum planum* (n = 12) and *Cystodytes lobatus* (n = 24) were treated as independent replicates. Non-parametric tests were used for the analysis of all results. Differences between the 2 ascidian species, with regard to epibacterial communities and settlement activities, were tested by the Mann-Whitney *U*-test. Correlations between epibacterial density, and antimicrobial and settlement activities, were calculated using the Spearman Rank Correlation Test. Level of significance was $p \leq 0.05$ (95 %).

RESULTS

Surface-associated bacteria: abundance and diversity

The numbers of culturable bacteria isolated from the surfaces of *Polyclinum planum* and *Cystodytes lobatus* were remarkably different (Fig. 2). The average number of bacteria calculated for *P. planum* was 15 534 cells cm^{-2} (SD ± 17 446, range 1224 to 65 520, n = 12). Bacterial densities on the surfaces of *C. lobatus* were low, averaging only 110 cells cm^{-2} (SD ± 114, range 0 to 418, n = 22). There was no overlap in the numbers of bacteria associated with the 2 species, with the least colonized *P. planum* featuring 3 times more bacteria per unit area than the most highly colonized *C. lobatus*. The differences in bacterial populations were extremely significant ($p < 0.0001$) with the average bacterial density on *P. planum* being 140 times higher than on *C. lobatus*. The striking difference in densities of bacteria

(as well as diatoms) on the 2 ascidian species is supported by SEM photographs (Figs. 3 & 4).

Bacteria isolated from *Cystodytes lobatus* and *Polyclinum planum* were categorized according to 11 criteria. Based on this analysis, bacterial diversity was similar on the 2 ascidian species. No specific bacterial associations or qualitative differences in the bacterial epiflora of the 2 species were evident, although it remains possible that these populations are distinct from bacteria in the surrounding seawater and adjacent epibiotic habitats, as has been demonstrated for marine sponges and algae (e.g. Wilkinson 1978, Lewis et al. 1985). The bacterial strains used in the settlement bioassay (PP1 and CL21-II) do not neccessarily represent strains that were abundant on the surfaces of *P. planum* and *C. lobatus*, nor were there obvious, specific associations between these strains and the ascidian species from which they were isolated.

Settlement effects

The extracts of *Cystodytes lobatus* and *Polyclinum planum* exhibited clear and specific effects on bacterial settlement (Fig. 5). *C. lobatus* extracts were highly deterrent, reducing bacterial settlement by the 2 test strains in 32 out of 34 cases (94 %). On average, *C. lobatus* extracts reduced settlement of strain PP1 by a factor of 5.13 (SE ± 0.8) relative to the control. The repellency of *C. lobatus* extracts is significant ($p < 0.01$) both with regard to the control and to *P. planum* extracts. Eleven of the *C. lobatus* extracts were tested against strain C21-II, and these extracts reduced settlement on average by a factor of 13 (SE ± 1.4). The reduction of settlement of strain C21-II by *C. lobatus* extracts, as compared to controls, is highly significant ($p < 0.001$). In the pilot study, an additional 8 out of 9
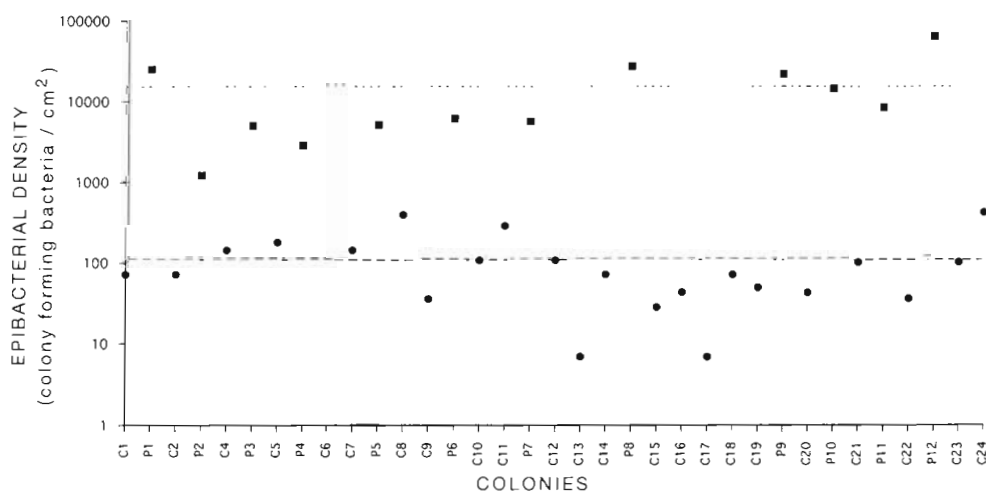

Fig. 2. Bacterial abundance on individual colonies of *Polyclinum planum* (P1 to P12, ■, n = 12) and *Cystodytes lobatus* (C1 to C24, ●, n = 24). Dashed lines are average abundances for *P. planum* (short dashes) and *C. lobatus* (long dashes). The average abundance of colony-forming units on *P. planum* is 140 times higher than on *C. lobatus*. This difference is extremely significant ($p < 0.0001$). The samples are shown in chronological order of collection
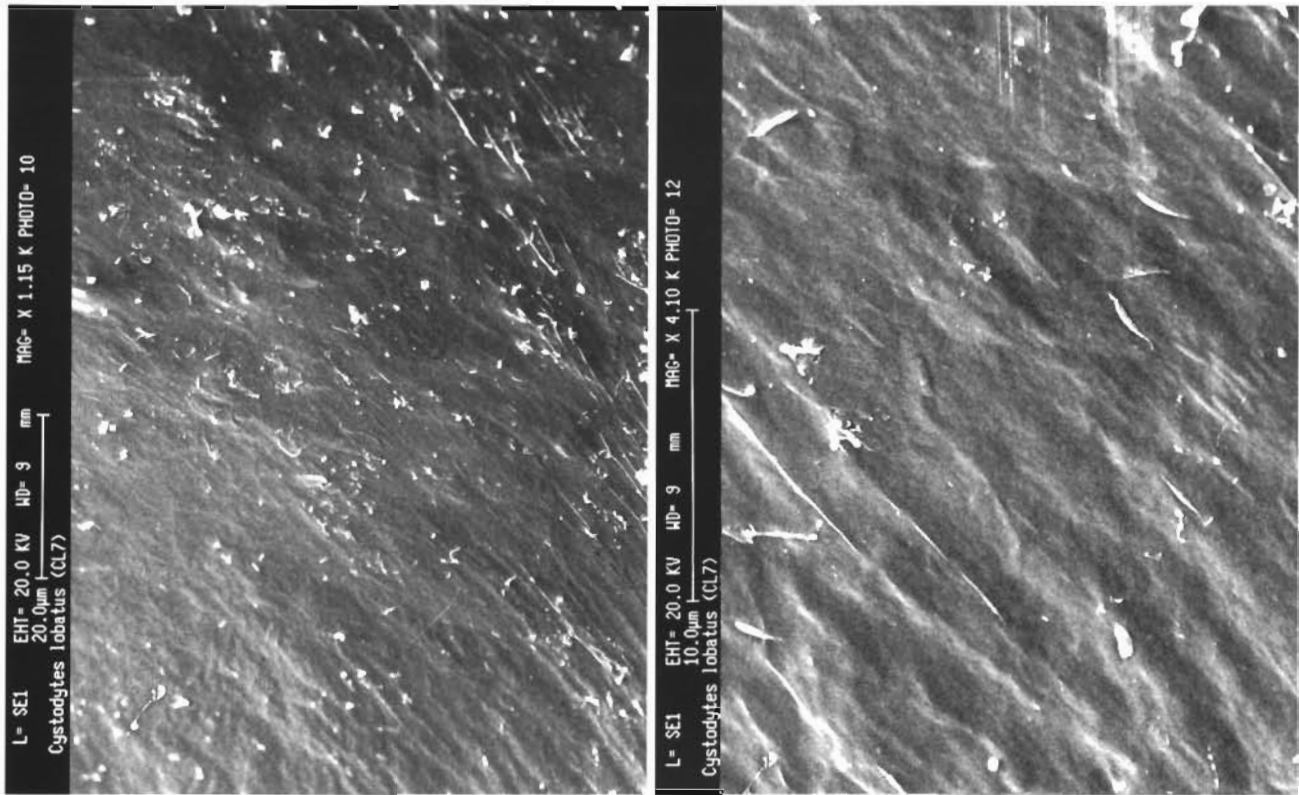
Fig. 4. SEM micrographs of *Cystodytes lobatus* surfaces showing typical *C. lobatus* colonies vitually free of epibiotic bacteria
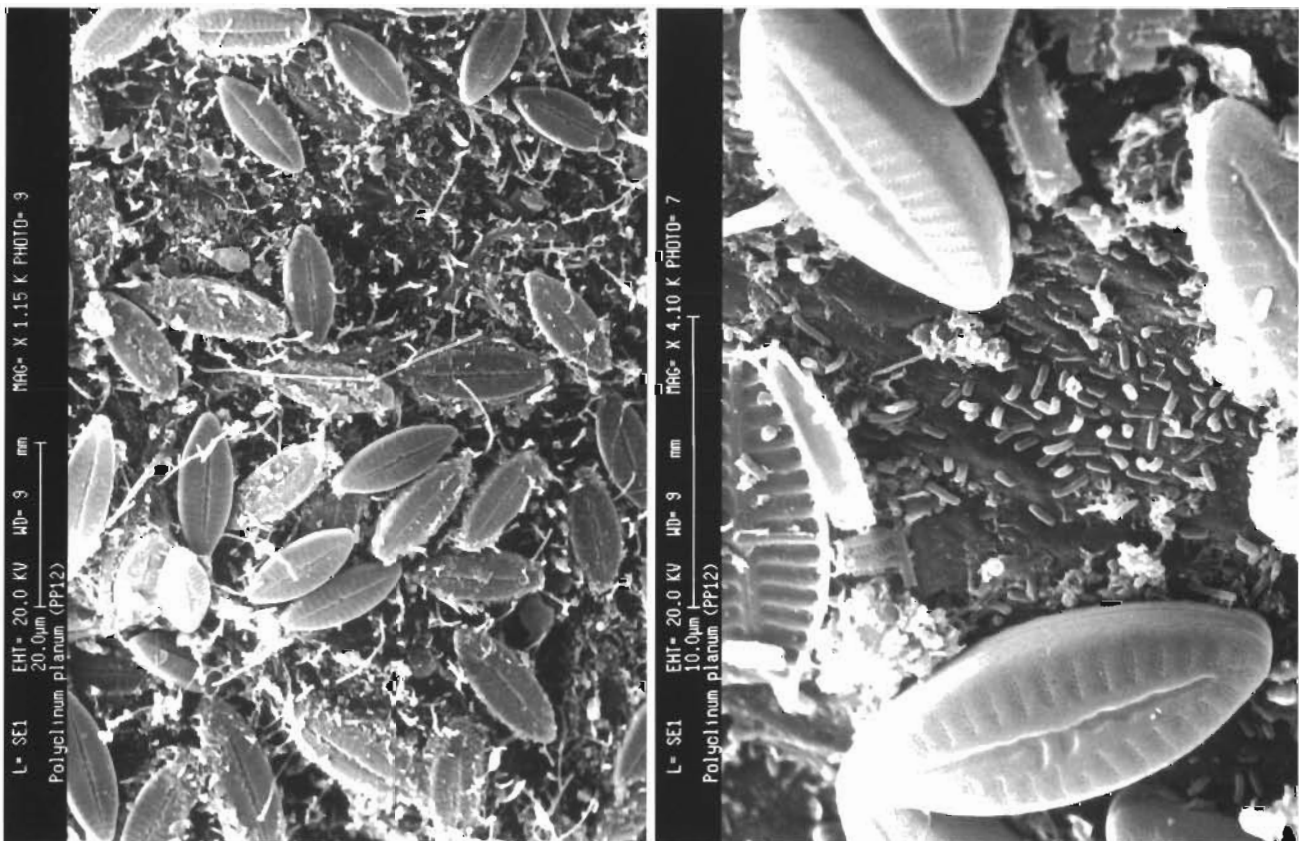
Fig. 3. SEM micrographs of *Polyclinum planum* surfaces featuring numerous diatoms and bacteria
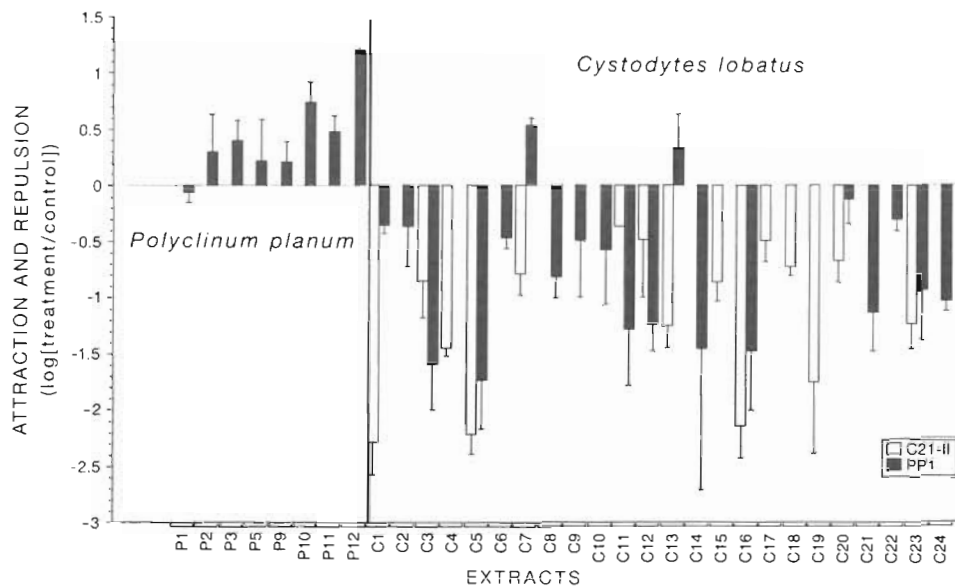
Fig. 5. Settlement bioassay: effects of *Polyclinum planum* (P1 to P12) and *Cystodytes lobatus* (C1 to C24) extracts on the attachment of bacterial strains PP1 (darkly shaded bars) and C21 and C21-II (lightly shaded bars) to the surface of an agar matrix. Results are shown for extracts replicated at least twice. Data expressed as [log (treatment/control) ± 1 SE]; >0 = attraction, <0 = repulsion

bacterial strains were strongly deterred from settling by *C. lobatus* extracts (Fig. 6), reducing settlement on average by a factor of 8.9 (SE ± 1.9).

In contrast to *Cystodytes lobatus, Polyclinum planum* extracts enhanced settlement by bacterial strain PP1, relative to the control, except for 1 extract that was essentially neutral (Fig. 5). Mean attraction was by a factor of 2.6 (SE ± 0.5). On average, bacterium PP1 settled 14 times more densely on substrata treated with *P. planum* extracts than on those treated with the extracts of *C. lobatus* (p < 0.0001). In the pilot study, pooled *P. planum* extracts attracted 9 out of 10 bacterial test strains on average by a factor of 3.6 (SE ± 0.8) com-

pared to the control. Considering all strains tested, settlement was 3 (versus strain TO3) to 300 times (versus strain AC2) higher on surfaces treated with *P. planum* extracts than on those incorporating *C. lobatus* extracts (Fig. 5). The difference observed is highly significant (p < 0.0001).

The surface extracts of 2 *Cystodytes lobatus* collections were separated into 3 solubility fractions by solvent partition, and these fractions were tested for settlement reducing activities. Subsequently, the active butanol soluble materials, further separated by silica-gel chromatography and HPLC fractionation, were also tested. Settlement reducing activity against
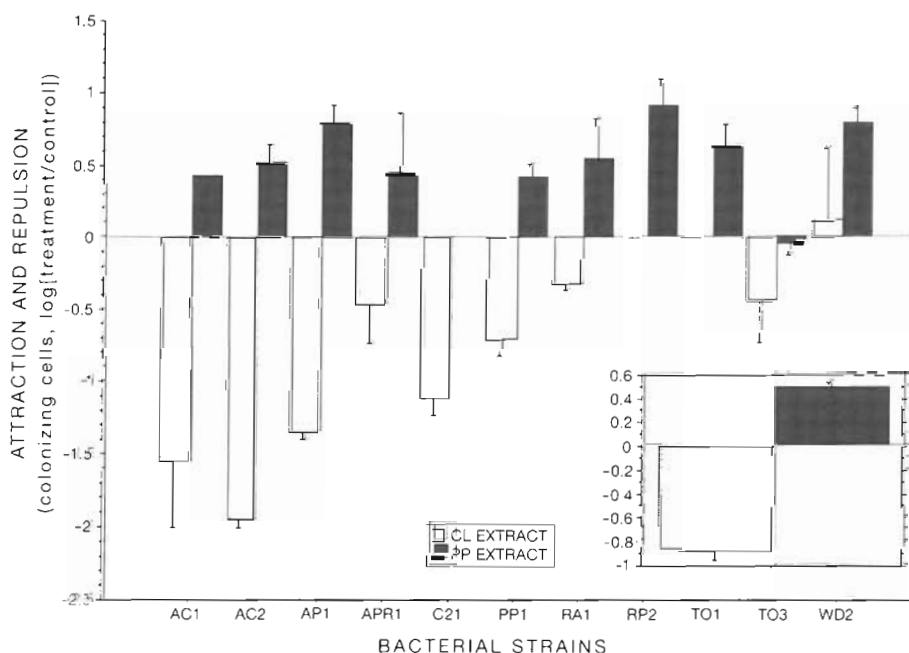

Fig. 6. Settlement bioassay in pilot study: effects of pooled *Polyclinum planum* (darkly shaded bars) and/or pooled *Cystodytes lobatus* extracts (lightly shaded bars) on settlement of 11 bacterial strains isolated from ascidian surfaces. Data expressed as [log (treatment/control) ± 1 SE]; >0 = attraction, <0 = repulsion. Inset: average antisettlement activity of CL and PP extracts (same *Y*-axis as main graph). Letters in strain code indicate ascidian host (for species abbreviations see Table 1)

Table 1 Pilot study: epibacterial abundance, antimicrobial activity, and antisettlement activity for 13 ascidian species. Conspecific ascidian colonies are pooled. CFU: colony-forming units; $X/\varnothing$: ratio of no. of cells on treated and control portions of microscope slide

Species	Epibacterial abundance (CFU cm^{-2})	Antimicrobial activity (area of no growth, mm^2)	Antisettlement activity	
			$X/\varnothing$	Log $(X/\varnothing)$
Aplidium californicum (AC)	13 000	0	0.25	−0.6
Aplidium propinquum (APR)	30 000	44	7.24	0.86
Archidistoma psammion (AP)	10 000	0	0.40	−0.4
Cystodytes lobatus (C)	500	0	0.20	−0.7
Didemnum sp. (white) (WD)	8 400	0	0.15	−0.84
Distaplia occidentalis (DO)	300 000	4	0.79	−0.1
Metandrocarpa dura (MD)	18 000	0	2.04	0.31
Polyclinum planum (PP)	450 000	10	4.07	0.61
Ritterella aequalisiphonis (RA)	360 000	0	1.00	0
Ritterella pulchra (RP)	16 000	0	1.45	0.16
Synoicum parfustis (SP)	68 000	0	0.40	−0.4
Trididemnum sp. (grey) (T)	1 700	0	0.40	−0.4
Trididemnum sp. (orange) (TO)	3 000	14	0.50	−0.3

strain PP1 was observed in the butanol soluble material (and to a lesser extent in the hexane soluble portion) of the crude extract, in fraction 11 of the VLC fractionation of the butanol soluble material, and in an HPLC purified substance derived from fraction 11.

Antimicrobial activity

The 12 replicate *Polyclinum planum* extracts were antimicrobially inactive against all 40 of the strains tested. In contrast, 21 of 24 *Cystodytes lobatus* extracts exhibited varying levels of antimicrobial activity against varying numbers of test strains. On average, extracts of single *C. lobatus* colonies exhibited antimicrobial activity against 42% (SD ±29%, range 0 to 80%) of the 40 bacterial strains tested. The average antimicrobial activity against these bacteria, measured as the area of no-growth around the paper disc, was 16 mm^2 (SE ±4.9, range 0 to 199 mm^2). All but 2 *C. lobatus* extracts inhibited *Bacillus subtilis* and all were inactive against *Escherichia coli*, *Candida albicans* and *Vibrio parahaemolyticus*.

In the pilot study (conspecifics pooled; Table 1), *Polyclinum lobatus* was weakly AB-active against one strain (AP1), but still favored its settlement (Fig. 6). On the other hand, *Cystodytes lobatus* extracts showed no activity against *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* or any of 9 strains isolated from ascidian surfaces, yet these same extracts showed potent settlement deterrence against these strains. It should be noted that strain C21-II, which was deterred from settling by all *C. lobatus* extracts tested, was insensitive to the antimicrobial effects of all *C. lobatus* extracts. In addition, 20% of the bacterial strains tested were

insensitive to all *C. lobatus* extracts and 3 *C. lobatus* extracts were inactive against all strains.

Extraction and chemical fractionation of the first *Cystodytes lobatus* collection resulted in the localization of the antimicrobial activity against strain PP1 within the aqueous fraction, and of the activity against *Bacillus subtilis* within 3 of the 14 VLC fractions (5, 12, 13) derived from the butanol fraction. The same fractions prepared from a second *C. lobatus* collection showed no antimicrobial activity against PP1, and weak activity against *B. subtilis* in 3 of the 14 VLC fractions (5, 6, 9). When compared to the results for the bioassay guided separation of the antisettlement activity, it is clear that the antimicrobial and antisettlement activities were located in different chemical fractions of the *C. lobatus* extract.

Correlations between epibacterial abundance and activities

On the intraspecific level, no significant correlation was found either between level of antisettlement activity and epibacterial abundance of single *Cystodytes lobatus* colonies, or between the level of extract antimicrobial activity (average of no-growth areas caused by a given extract versus all test strains) and epibacterial abundance on the surface of the *C. lobatus* colony that yielded the extract ($p > 0.1$, $n = 24$). Nor was there any correlation between the reduction in PP1 settlement and the level of antimicrobial activity in *C. lobatus* colonies ($r_S = -0.1$, $p > 0.25$, $n = 24$).

On the species level (incorporating the results of the pilot studies, Table 1), strong antisettlement activity of an ascidian species was correlated with reduced epibacterial abundance on this species ($n = 13$ species,

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p = 0.02, r_S = 0.67), while antimicrobial activity was not (n = 13 species, p = 0.98, r_S = 0.006).

DISCUSSION

Organic substances produced by marine plants and animals have been shown to affect bacterial behavior (e.g. Bell & Mitchell 1972). The extent to which secondary metabolites function as an antibacterial chemical defense, however, has not been demonstrated (Bakus et al. 1986, Paul 1992). The lack of evidence for the existence of antibacterial chemical defenses in marine systems is surprising, considering the widespread *in vitro* occurrence of antimicrobial activity in extracts of marine plants and invertebrates (Rinehart et al. 1981). Although these activities may represent antibacterial defense mechanisms, a correlation between laboratory-based antimicrobial activity and *in situ* effects upon marine bacteria has not been demonstrated. Due to this lack of experimental evidence, the ecological roles of marine metabolites, in regard to their effects upon marine bacteria, remain speculative.

We studied the bacterial populations associated with the surfaces of 2 marine ascidians, *Cystodytes lobatus* and *Polyclinum planum*. These co-occurring ascidians exhibit remarkable quantitative differences in epibacterial populations. The surfaces of *C. lobatus* displayed extremely low numbers of bacteria relative to *P. planum*, and relative to what would be expected for a submerged surface in general. As can be seen from SEM photographs (Fig. 4), the surfaces of *C. lobatus* are virtually free of attached bacteria. Extracts of *C. lobatus*, imbedded in agar matrices at concentrations below those estimated to occur in the species, were found to dramatically deter bacterial settlement. These results provide the first demonstration that naturally occurring organic metabolites, extracted from a marine invertebrate, can inhibit the attachment of bacteria to a surface.

In contrast to *Cystodytes lobatus*, the surfaces of *Polyclinum planum* exhibited dense epibacterial populations (Fig 3). The numbers of bacteria settling on agar matrices incorporating extracts of *P. planum* were significantly greater than those settling on controls. The chemical attraction of bacteria to extracts of *P. planum* is thus offered as an explanation for the relatively high bacterial abundance on the surface of this species. As is evidenced by the deterrent effect of *C. lobatus* extracts, this role may include that of a chemical defense against bacterial settlement. On the species level (comparison of 13 ascidian species), the correlation between chemically mediated effects on bacterial settlement and surface bacterial abundance was significant. This supports the contention that host

metabolites play an important role in controlling surface bacterial populations.

The extracts of *Cystodytes lobatus* exhibited both settlement reducing and antimicrobial effects. After assessing the results obtained for these bioassays, we suggest that effects on bacterial settlement are better suited than antimicrobial activity to explain the low numbers of bacteria associated with *C. lobatus*. In defense of this proposal we argue that (1) in *C. lobatus* extracts settlement reduction was the dominant activity (94 vs 42% active interactions), and 9 out of 10 antimicrobially insensitive strains (including C21-II) were deterred from settling by *C. lobatus* extracts, (2) the antimicrobial activities of *C. lobatus* extracts were inconsistent among samples and were in no case statistically correlated to settlement reducing activities or epibacterial abundance, and (3) the antimicrobial and settlement reducing activities were observed in different, chemically-distinct fractions of the crude extracts suggesting that they are caused by different metabolites. Thus, these 2 activities can be considered independent, demonstrating that the observed bacterial settlement deterrence is not due to antimicrobial toxicity. This is not to say that antimicrobial substances do not perform a defensive role against microbial colonization. It is tempting to hypothesize that antisettlement and antimicrobial activities may act in complementary ways, e.g. bacteriostatic compounds inhibiting the growth the few cells that attach in spite of the antisettlement activity. Alternatively, or in addition, antimicrobial activity could be a defensive adaptation targeted against specific pathogens. Also, the 2 distinct activities in *C. lobatus* might constitute a multiple defense system more difficult to bypass coevolutionarily.

The results presented here are in agreement with the view that bioassays designed to derive pharmacologically relevant information may not be good predictors of the ecological function of secondary metabolites (Hay et al. 1987, Davis 1991). The observation that antimicrobial activity poorly correlated with surface bacterial abundance even when all results were pooled (13 species) may be due to the fact that bacteria can be affected by host metabolites at any phase of the colonization process, and by a number of different mechanisms. The measurement of 'antimicrobial activity', indicating bacteriocidal or bacteriostatic effects, reflects only 1 mechanism (the inhibition of bacterial growth) by which epibacterial abundance can be affected. Bacterial colonization of a surface may be influenced by additional factors including chemotaxis (attraction or repulsion) and factors affecting cell motility and the production of extracellular polymers used for irreversible attachment. The settlement bioassay, as employed here, integratively screens for effects on

the first 2 phases of colonization (approach and attachment), and affords bacteria the opportunity to respond, chemotactically, to the extract. This is especially important considering that negative chemotaxis can be induced in marine bacteria by non-toxic concentrations of organic substances (Chet et al. 1975).

The settlement bioassay was not designed to account for either the conspicuously reduced diatom fouling on *Cystodytes lobatus* (Figs. 3 & 4), or the observed absence of larger fouling organisms on both species. These features may also be due to chemical deterrence, as secondary metabolites have been shown to inhibit diatom growth (Bandurraga & Fenical 1985), and have been linked to observations of reduced macro-fouling (Davis 1991). To investigate these questions in *Polyclinum planum* and *C. lobatus*, the settlement bioassay could be modified to allow longer colonization times (e.g. the use of a slow-leaching matrix) which would permit testing of non-bacterial fouling organisms (e.g. diatoms, invertebrate larvae), and *in situ* testing of extracts (e.g. Kangatharalingam et al. 1990). In addition, some of the *C. lobatus* colonies, in contrast to *P. planum*, were observed to secrete mucus when handled. While this event was rare and seemed to be triggered by handling, the possiblity remains that mucus secretion might constitute a mechanical component to the antifouling control in this species.

The crude extracts tested in this study were complex mixtures of primary and secondary metabolites. Some of these may be nutrients and, as a consequence, may attract motile bacteria (Chet & Mitchell 1976). It is possible that the increased settlement of bacteria on *P. planum* extracts is due to attraction of bacteria to a nutritionally favorable environment, although it has been shown that there is no correlation between metabolic value of a substrate and its ability to attract bacteria (Chet & Mitchell 1976).

Considering that in the settlement bioassay only 2 strains of bacteria were tested against the replicated extracts (and an additional 7 to 9 strains against the non-replicated extracts), little can be concluded about the overall responses of marine bacteria to extracts of *Polyclinum planum* and *Cystodytes lobatus*. Strains PP1 and CL21-II were chosen for the bioassay, in part, because they were isolated as epibionts of *P. planum* and *C. lobatus*. The effects of ascidian extracts upon the settlement of PP1 and CL21-II are made more meaningful given the *in situ* ability of these strains to attach to ascidian surfaces. There was no indication that either PP1 or CL21-II were specific to, or numerically dominant upon, the ascidian from which they were isolated. These strains appear to represent typical bacteria that can be isolated from the surfaces of *P. planum* and *C. lobatus*, however, the extent to which they differ from strains inhabiting neighboring micro-

habitats has not been determined. Explanations as to why strain C21-II was isolated as an epibiont of *C. lobatus*, when it is strongly deterred from settling on surfaces incorporating extracts of this species, can only be speculated upon at this time. However, it is clear from the total numbers of bacteria observed on the surface of *C. lobatus*, that it is extremely uncommon for C21-II, or any other strain of bacteria, to settle on the surface of this ascidian. Future studies, employing a greater variety of marine bacteria, and addressing the distributions and possible exudation of settlement deterring substances, may help answer some of these questions.

From the data presented here, it can be concluded that extracts of *Polyclinum planum* and *Cystodytes lobatus* affect the numbers of bacteria that attach to an agar surface. The chemical enhancement or inhibition of bacterial settlement was correlated with epibacterial abundance on 13 ascidian species and may, possibly in cooperation with the more erratic antimicrobial activity in *C. lobatus*, offer an explanation for the dramatically different numbers of bacteria observed on the surfaces of *C. lobatus* and *P. planum*. These results support the hypothesis that invertebrate metabolites are involved in the regulation of microbial distributions in the marine environment, and may act as a chemical defense aimed at controlling surface bacterial colonization.

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