

Antimicrobial activity and surface bacterial film in marine sponges

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Abstract

The antimicrobial activity of three sponge species was tested against marine benthic bacteria and the presence of epibiotic bacteria on their surfaces was investigated. The aim of the present study was to determine whether there is a correlation between antimicrobial activities and the presence of a bacterial film. Seven benthic bacterial strains were isolated from the vicinity of the sponges and used as assay organisms. Gram-positive and Gram-negative bacteria were equally affected by all the sponge extracts. The encrusting sponge *Crambe crambe* featured the strongest antimicrobial activity in the assays and no bacteria were found on its surface. The other two sponges, *Ircinia fasciculata* and *Spongia officinalis*, featured lower antimicrobial activity than *C. crambe* and the number of bacteria found on their surfaces was of the same order of magnitude as that found on immersed glass slides used as controls. It was concluded that antimicrobial activities detected in laboratory assays were effective as mechanisms to combat microfouling in only some cases, and other possible interpretations are considered.

Key words: Antimicrobial activity; Bacterial film; Fouling; Sponge

1. Introduction

Bare substratum is usually scarce in rocky littoral communities, and constitutes a limiting resource for benthic organisms (Jackson & Buss, 1975). As a consequence, epibiosis is a widespread phenomenon in these environments. Although fouling seems likely to be disadvantageous to the basibionts, a wide array of responses to this phenomenon can be found in nature. On the one hand, basibionts may tolerate different

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degrees of epibiosis and even total overgrowth (Rützler, 1970; Dayton, 1971). They may, in fact, benefit from these fouling organisms (Stoecker, 1978; Fishlyn & Phillips, 1980; Osman & Haugness, 1981; Bakus et al., 1986; Dyrinda, 1986). On the other hand, many organisms do not tolerate epibiosis, but respond to it. An array of different mechanisms have been selected as antifouling defences, such as special surface structures (e.g. protruding spicules), continuous surface renewal, mucus production, shedding of the epidermis or production of toxic compounds (Bakus et al., 1986; Dyrinda, 1986; Davis et al., 1989; Wahl, 1989; Wahl & Banaigs, 1991; Turon, 1992). Organisms resorting to one or more of these defence mechanisms may be free of epibionts. Although epibiosis is not necessarily advantageous or disadvantageous for either partner (Wahl, 1989), if any living immersed surface is free of epibionts, it must be as a consequence of some mechanism(s) preventing fouling.

The role of toxic compounds as antifouling mechanisms has become apparent in recent years. These compounds must deter the settlement of organisms (Davis & Wright, 1990; Davis et al., 1991) or kill them when they have just settled by forming an unfavourable microenvironment. There is controversy as to whether the formation of a bacterial film is necessary for subsequent biofouling of a surface, although it seems clear that the bacterial film can enhance or inhibit further stages of colonization (Bakus et al., 1990; Davis et al., 1989; Clare et al., 1992). In spite of this, a negative correlation has been found between antimicrobial activity and the percentage cover of fouling organisms (Davis & Wright, 1989; McCaffrey & Endean, 1985; Thompson et al., 1985; Uriz et al., 1992), though no research has specifically addressed whether the antibacterial activities detected in the laboratory have a role as an antifouling mechanism.

The purpose of this study is to compare the antifouling abilities of three species of Demospongiae: *Crambe crambe* (Schmidt, 1862) (Poeciloscleridae), *Ircinia fasciculata* (Pallas, 1766) (Thorectidae) and *Spongia officinalis* Linné, 1759 (Spongiidae). The former two were known to have antibacterial properties, while the latter species did not display antibacterial activity (Burkholder & Ruetzler, 1969; Amade et al., 1987; Huysecom et al., 1990; Uriz et al., 1992). Our purpose was to investigate whether species with antimicrobial activities featured epibiotic bacteria. In order to avoid the indetermination caused by the normal procedure of testing activities against terrestrial forms or against bacteria that are not found in the sponges' environment, we performed tests on bacteria collected in the vicinity of the specimens studied. We also determined the number of bacteria settled on the surface of naturally-occurring specimens of these sponges. In the community studied, the species *C. crambe* was always found completely free of macrofouling, while *S. officinalis* and *I. fasciculata* were occasionally found with macroepibionts.

2. Materials and methods

All the specimens studied in this work inhabited the same photophilic community (Becerro et al., 1994) located in Blanes (northwestern Mediterranean; 41° 40.4'N, 2° 48.2' E) and were collected in summer from a vertical wall at a depth of 5 m by SCUBA diving.

2.1. Extraction procedure

Several (from 5 to 7) specimens of each species were collected, rinsed in distilled water, freeze-dried and ground together. It was necessary to join several specimens in order to obtain sufficient amount of extract. The samples were then extracted with acetone (e.g. Cimino et al., 1993) in a proportion of 0.1 g dry weight in 10 ml of solvent. The solvent was evaporated under reduced pressure and the residue was weighed and dissolved in distilled water to obtain a concentration of $5 \text{ mg} \cdot \text{ml}^{-1}$.

2.2. Antimicrobial assay

A total of seven bacterial strains were isolated from the sediment near the sponges in order to test the antimicrobial activity of the sponges under investigation. Six-millimeter sterile paper disks were soaked with $5 \mu\text{l}$ of the solutions previously described to give a final disk loading of $25 \mu\text{g}$ of crude extract (preliminary tests showed that this loading allowed accurate activity readings). Disks were placed on agar plates previously seeded with the bacterial strains. Four replicates were used for each treatment. The diameter of the inhibition zone (including the disk) was measured to the nearest mm after 24 h at 30°C .

2.3. Bacterial isolation

Five specimens of each species were separately introduced underwater into a glass pot containing sterile seawater ($0.2 \mu\text{m}$ filtered seawater). Once in the laboratory, the sponges were separately placed in sterile jars with $300\text{--}400 \text{ cm}^3$ sterile seawater that was changed four times in order to remove free bacteria in the water and on the surface. The same treatment was applied to three glass slides which were previously immersed for 4 wk in the study area as controls.

In the case of *I. fasciculata* and *S. officinalis* a zone free of macroepibionts was selected for bacterial isolation, since these fouling organisms would contaminate both the water and the surface of the sponge.

Bacteria were obtained from the surface of the sponges (and glass slides) following the method of Wahl & Lafargue (1990). A small area of each specimen (1 cm^2), at least 1 cm from the border, was softly swabbed with a sterile cotton-tipped pipette. The cotton-tipped end of the pipette was placed in a 50 ml glass vessel containing Ringer solution. Solutions were cultured in Marine Agar 2216 (DIFCO) after shaking and making log dilutions. The numbers of colonies were counted after 24 h at 30°C . This temperature is slightly higher than the summer water temperature in this region ($23\text{--}25^\circ\text{C}$). The Petri dishes were kept in incubation for 13 days to make sure that no significant changes occurred in the number of bacteria appearing in the treatments that were devoid of them at 24 h.

We also checked whether the bacteria obtained by this procedure were true surface bacteria or were taken from the surrounding water or from the mesohyl of the sponge. The sterile water used in the washings may have been contaminated by bacteria present in the aquiferous system of the sponges, while mesohyl bacteria may have been taken

in the case of imperceptible damage inflicted to the sponges' pinacoderm. For this purpose, the most delicate of the three sponges studied, *C. crambe*, was used to check the number of bacteria in the water from the final washing and in its inner part. Bacteria from the *C. crambe* choanosome were obtained by cutting the sponge with a sterile scalpel and swabbing the two resulting surfaces. By this procedure free bacteria from the aquiferous system may also be taken, and the results obtained must be checked against those from the surrounding water. Bacteria from the water were obtained from 10 μ l of the last washing with sterile water (see above). The cotton and the 10 μ l were then placed in the Ringer solution, which was log diluted and cultured as previously described. An overall control was performed in the same way by swabbing a sterile Petri dish.

Since bacteria isolated from the choanosome of *C. crambe* may be symbiotic, antimicrobial assays were also run with the *C. crambe* extract to test their resistance.

2.4. SEM methods

For SEM observation, several pieces of the sponges used in the bacterial isolation were cut from the zones which had not been swabbed. They were then freeze-dried, coated with gold (sputtering method) and observed under a Cambridge S-120 microscope.

2.5. Numerical methods

Since the assumptions of normality and homoscedasticity were not met by our data even after trying several transformations (as tested by Kolmogorov-Smirnov and Bartlett tests, respectively), we used non-parametric procedures (Mann-Whitney and Kruskal-Wallis tests). Post-hoc non-parametric tests were performed using the Dunn method for unbalanced data (Zar, 1984).

3. Results

The three sponge species studied displayed antimicrobial activity, as shown both by the number of bacteria inhibited and by the diameters of the inhibition zone. The overall means of the inhibition diameters (Table 1) featured marked differences among sponge species (Kruskal-Wallis statistic = 21.112, $df = 2$, $p < 0.001$). Post-hoc comparisons showed that the strongest antimicrobial activity corresponds to *C. crambe*, which was significantly higher than that displayed by *I. fasciculata* ($p < 0.001$) and *S. officinalis* ($p < 0.05$). Furthermore, *C. crambe* featured bacterial inhibition against all the bacteria used (Table 1). There were no significant differences in the inhibition zone between *I. fasciculata* and *S. officinalis* ($p > 0.2$), although the former was active against six of the bacteria strains assayed and the latter was active against only four (Table 1).

Mann-Whitney U-tests between Gram-positive and Gram-negative bacteria yielded no significant differences for any sponge (Table 2).

With regard to the presence of a bacterial film (Table 3), a highly significant treat-

Table 1

Inhibition of bacterial growth by sponge extracts. Mean and SD calculated from four replicates for each species

Bacterial strains		Sponge		
Code	Type	<i>C. crambe</i>	<i>I. fasciculata</i>	<i>S. officinalis</i>
B1	Gram +	9.5 ± 1.2	NT	NT
B2	Gram +	20.5 ± 1	11 ± 1.1	NT
B3	Gram +	18.7 ± 0.5	7.2 ± 0.5	9.5 ± 0.5
B4	Gram +	18 ± 2.1	7.2 ± 0.5	NT
B5	Gram -	24.2 ± 0.9	10.5 ± 0.5	8.5 ± 0.5
B6	Gram -	12.5 ± 0.5	7.2 ± 0.5	14.2 ± 4.5
B7	Gram -	7.7 ± 0.5	10.5 ± 1.2	10.7 ± 1.8
Overall mean		15.89	8.95	10.75
95% c.i.		2.19	0.77	1.60

Overall mean diameter of inhibition (mm) and 95% c.i. are also displayed (only strains for which a toxicity was found were included in these means). NT = non toxic activity.

ment effect was found on the density of bacteria obtained (Kruskal-Wallis statistic = 56.56, df = 4, $p < 0.001$). Indeed, very few isolates were found on the surface of *C. crambe* and the sterile controls, in contrast to the abundance of bacteria found in the cultures from the immersed glasses and the surfaces of *I. fasciculata* and *S. officinalis*. The abundance of bacteria on *C. crambe* surfaces featured no significant differences in number of bacteria ($p > 0.5$) with the sterile control, while showing significantly lower numbers (at the 0.05 probability level) than the immersed glasses and the other two species. The number of bacteria found on *I. fasciculata* and *S. officinalis* did not differ from each other nor with respect to the immersed glasses ($p > 0.5$ in both cases). After 13 days, the cultures from the sterile controls and the surface of *C. crambe* had the same axenic appearance. The SEM images also showed a epibiont-free surface in *C. crambe*, while variable amounts of micro-organisms appeared in the other two species (Figs. 1–6).

Table 2

Summary of Mann-Whitney *U*-tests performed for means of diameter inhibition between Gram-positive and Gram-negative bacteria by the three sponge species studied

Sponge	Group	<i>N</i>	Mean	SD	Mann-Whitney <i>U</i> -test	<i>p</i>
<i>C. crambe</i>	Gram (+)	16	16.688	4.557	110.5	0.499
	Gram (-)	12	14.833	7.272		
<i>I. fasciculata</i>	Gram (+)	12	8.500	1.977	52.0	0.232
	Gram (-)	12	9.417	1.782		
<i>S. officinalis</i>	Gram (+)	4	9.500	0.577	18.0	0.460
	Gram (-)	12	11.167	3.589		

Table 3

Mean ($\times 10^4 \times \text{cm}^{-2}$) and 95% c.i. of the bacterial densities encountered in the different treatments studied

Treatment	N	Mean	95% c.i.
Sterile control	5	0.003	0.005
Immersed glass	3	1.894	0.656
<i>C. crambe</i>			
Water	2	28.500	5.542
Choanosome	2	1.000	0.800
Surface	5	0.006	0.008
<i>I. fasciculata</i>	5	3.480	0.925
<i>S. officinalis</i>	5	3.653	0.528

The cultures from both the last washing and the choanosome of *C. crambe* showed high numbers of bacteria in the water, while only a few were isolated from the choanosome of the sponge. In the antimicrobial test run with the latter, two out of five bacterial strains were resistant to the extract (Table 4). One of these (Choan-1) was not affected by the sponge extract whilst the other (Choan-2) displayed an inhibition zone in which the bacteria were not killed but had a much lower density than outside the zone of influence.

4. Discussion

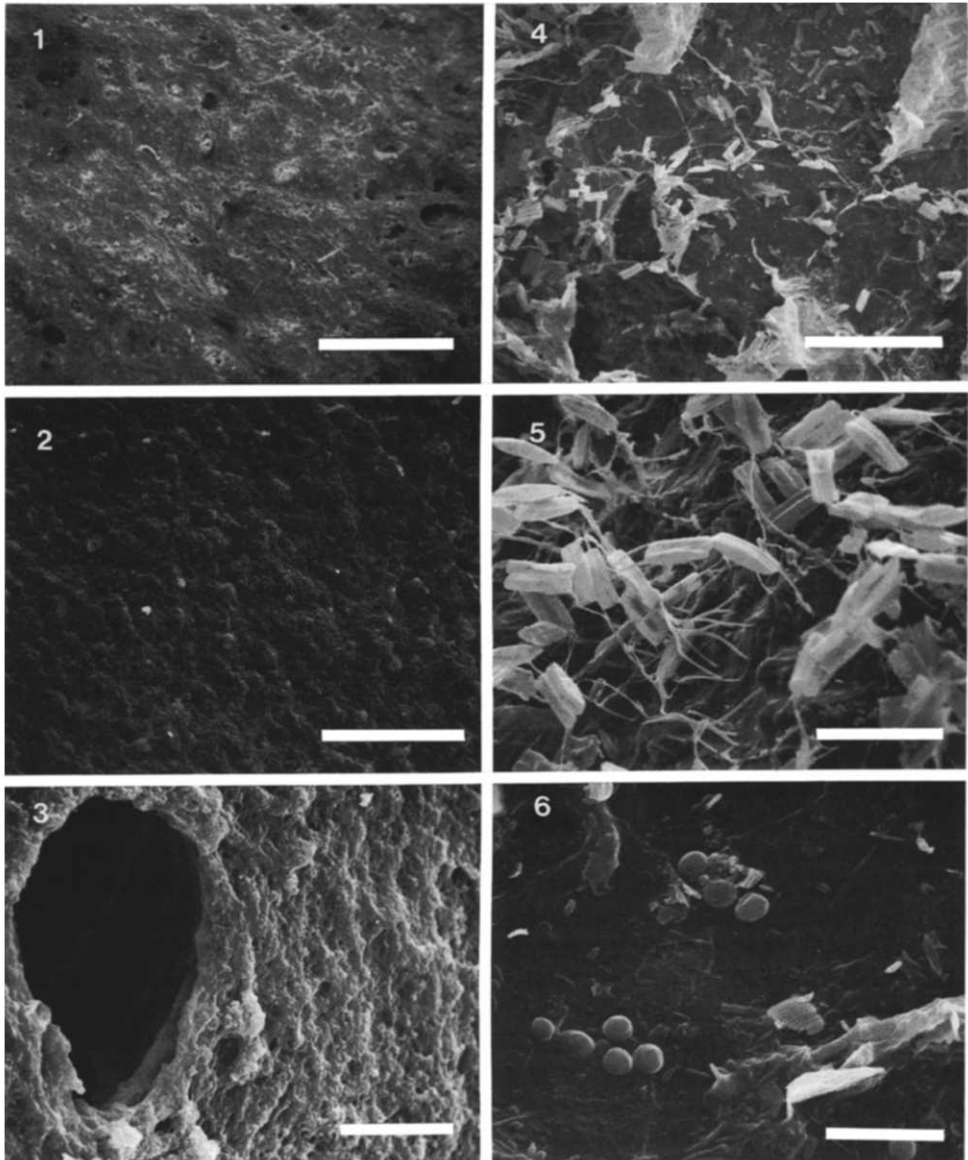
The antimicrobial activities of *C. crambe*, *I. fasciculata* and *S. officinalis* have been previously tested by Burkholder & Ruetzler (1969), Amade et al. (1987) and Uriz et al. (1992). In all cases the activity was determined by the paper disk diffusion method (Burkholder & Ruetzler, 1969). In this study the antimicrobial activity found was stronger than that reported in these references (indeed antimicrobial activity for *S. officinalis* is not described in these references), although we used a much smaller amount of crude extract. There may be several explanations for these results. First, the different extraction procedures; second, natural variation in the amount of toxic compounds

Table 4

Resistance to the extract of *C. crambe* by the five bacterial strains isolated from its inner part

Bacteria	Mean	SD
Choan-1	No inhibition	
Choan-2*	10.75	0.5
Choan-3	14.75	0.5
Choan-4	12	1.41
Choan-5	21	2.16

Diameter of inhibition zone in mm. * Inhibition zone with much lower bacterial density.



Figs. 1–6. SEM images of surfaces of *C. crambe* (Figs. 1–3) and *I. fasciculata* (Figs. 4–6) at different magnifications, showing its axenic nature in the former species, and the abundance and diversity of microepibionts in the latter. Scale bars: Figs. 1 and 4 = 200 μm . Figs. 2 and 5 = 50 μm . Fig. 3 = 10 μm . Fig. 6 = 5 μm .

among individual sponges, and finally, the diverse responses of the different assay organisms used in the toxicity tests.

Differences in the extraction procedure may lead to a bias in the compounds in the

crude extract. Even if the active molecules are extracted to the same extent, the procedure followed may affect the extraction of other compounds that could have synergistic effects (Rinehart et al., 1987).

Variation in toxicity among specimens may also be expected, since toxic compounds are secondary metabolites and can be affected by changes in the metabolism of the species. Differences in chemicals production by a given species have already been reported in the marine environment and have been attributed to variation in biological and ecological characteristics, such as location, age, predation pressure and intra-individual variability (Thompson et al., 1985; Coll et al., 1987; Paul & Van Alstyne, 1988, 1992). For the present purposes, the need for a certain amount of extract to perform the tests precluded the analysis of toxicity and epibiosis on the same specimens, which might have allowed direct comparisons. However, as we ground several specimens together to obtain the extract, individual variation may have been smoothed, thus giving an accurate estimate of the toxicity of the species, rather than the individuals. Estimating the toxicity at the species level may still provide a sound basis for interpretation. On the other hand, previous studies by the authors (Uriz et al., 1991, 1992; Martin & Uriz, 1993, and unpublished results) have shown that intraspecific differences in activity, while they may have great ecological relevance, are usually small. Whenever a species was found to be active, all the specimens of this species tested displayed activity to some degree.

As regard to the different assay organisms used in the toxicity tests, it is obvious than the specific characteristics of an organism will determine its response to toxic compounds. Testing ecological hypotheses on marine species by using terrestrial assay organisms or even marine ones that are not present in the communities considered is arguable (Berquist & Bedford, 1978; Bakus et al., 1990). Care was taken in this study to use only bacteria living in the same community and in the vicinity of the sponges.

There is some controversy as to whether Gram-positive (Burkholder & Ruetzler, 1969; McCaffrey & Endean, 1985) or Gram-negative (Berquist & Bedford, 1978) bacteria are more affected by the antimicrobial compounds of sponge extracts. In this study, both kinds of bacteria were affected to the same degree by the extracts of the three sponges studied. Our results are coincident with those of Amade et al. (1982). The variations in the type of bacteria sensitive to sponge compounds may be due to the different sponge species studied (Amade et al., 1987) and the different species of bacteria used in the tests.

We found significant differences in the antimicrobial activity of *C. crambe* compared to *S. officinalis* and *I. fasciculata*. We also found that bacteria, and occasionally macrofoulers, were present on the surface of the latter two species, while *C. crambe* featured a near axenic surface. SEM observation supported the results obtained in the cultures. The data are valid for comparisons among the species studied and are not intended for absolute quantifications, which are beyond the scope of this work. Although the plate culture temperature used was higher than the warmest sea temperatures in this area, previous studies (Lopez, 1993) showed that these conditions speed up the development without killing the benthic bacteria of this area. At this temperature, 24 h incubation time allowed easy quantification of the bacteria present. At longer times the bacterial colonies began to overlap and became difficult to count. It is reas-

sure, in this sense, that no appreciable numbers of bacteria appeared in the cultures from *C. crambe* even after 13 days of incubation.

We found high densities of bacteria in the (originally sterile) water used in the last washing from *C. crambe*, but apparently they did not have a significant effect on our analyses since almost no bacteria were collected from the surface of this species. Very few isolates were obtained from the inner part of *C. crambe*. Two bacterial strains isolated from this sponge zone seemed to be resistant to the antibacterial compounds from the same species, which nicely adjusts to what would be expected for symbiotic forms. However, because of the low number of bacteria isolated, the possibility that they represent contamination cannot be ruled out.

The lack of epibionts on *C. crambe* agrees well with the strong antibacterial activity displayed by the sponge extracts. Of course, no causal evidence can be inferred from these correlational data. However, no mechanical defence, mucus production or sloughing has either been described or detected in this species, suggesting a true chemically mediated inhibition (unless other mechanisms of physical defence not usually considered were acting, i.e. Ballentine, 1979; Wahl, 1989).

On the other hand, in spite of the antibacterial activities encountered, *I. fasciculata* and *S. officinalis* featured numbers of bacteria per cm² as high as the immersed glass slides which served as a control. The simplest explanation would be that substances obtained by an unavoidably artificial extraction procedure may never come into contact with epibionts in the field and actually play other roles in the sponge's metabolism, their activities in vitro lacking any antifouling function. Alternatively, the presence of these bacteria on their surface might be the result of a co-evolution with the sponge, during which resistant strains could have been selected. Furthermore, it is also possible that these epibiotic bacteria are actually responsible for the antimicrobial activity detected in *S. officinalis* and *I. fasciculata*.

In conclusion, although subject to some limitations (only chemical compounds extracted with acetone were tested, and the bacteria used were not actually epibionts of the sponges), the evidence gathered supported that antibacterial compounds detected in laboratory assays may play an ecological role as antifouling mechanisms, as in the case of *C. crambe*. However, other functions must also be envisaged. Endobacterial population control (Amade et al., 1987), predator deterrence (Green, 1977; Thompson, 1985), competition for substrata (Uriz et al., 1992) or enhancement of the filter-feeding efficiency (Bergquist & Bedford, 1978) may be also performed through these compounds, allowing an interpretation of the apparently contradictory results obtained for *I. fasciculata* and *S. officinalis*.

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