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Characterisation and antimicrobial activity of epibiotic bacteria from *Petrosia ficiformis* (Porifera, Demospongiae)

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Abstract

Numerous natural products from marine invertebrates show striking structural similarities to known metabolites of microbial origin, suggesting that microorganisms (bacteria, microalgae) are at least involved in their biosynthesis or are in fact the true sources of these respective metabolites.

The viable epibiotic microbial community of the marine sponge *Petrosia ficiformis* was screened and characterised using classical and molecular techniques. Fifty-seven aerobic heterotrophic bacterial strains were isolated and presumptively identified by their phenotypic characters. Random Amplified Polymorphic DNA (RAPD) analysis of the non-fermentative Gram-negative strains and Corynebacteria was performed. Cluster analysis of RAPD data showed genetic relatedness among sponge isolates. Antimicrobial activity was found in several isolates, two of which were identified as *Rhodococcus* sp. and *Pseudomonas* sp. by partial 16S rRNA gene sequencing. The recovery of strains with antimicrobial activity suggests that marine sponges represent an ecological niche which harbours a largely uncharacterised microbial diversity and a yet unexploited potential in the search for new secondary metabolites.

Keywords: Antimicrobial activity; Sponge-associated microorganisms; RAPD; *Petrosia ficiformis*

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1. Introduction

The study of marine bacteria and their potential role in the production of metabolites is becoming a new topic for research (Faulkner, 2000). Several investigations have supplied an increasing number of biologically active and/or structurally unique compounds (Barsby et al., 2001; Hardt et al., 2000). Although major attention has been dedicated to the study of microorganisms of shallow and deep-water sediments, marine bacteria able to produce antibacterial and/or antifouling metabolites were prevalently found in association with marine invertebrates and plants (Holmström and Kjelleberg, 1999; De Rosa et al., 2000; Egan et al., 2000). Here, they may promote survival and competition especially for sessile hosts which base their passive defence on chemical and/or physical devices. As far as sponges are concerned, up to 40% of their biomass can be constituted by microorganisms (Wilkinson, 1978).

Sponges can host heterotrophic bacteria, Archaea, Cyanobacteria and unicellular algae (Hentschel et al., 2001), many of which play a role in sponge nutrition, metabolic transport (Wilkinson and Garrone, 1980), and defence against predators and biofouling (Unson et al., 1994; Bewley et al., 1996). Even though the mesohyl microbial community has been extensively studied (Wilkinson, 1978; Vacelet and Donadey, 1977; Friedrich et al., 1999; Olson et al., 2002), the description of surface microbial communities of marine sponges is scarce (Becerro et al., 1994), while biofilms could account for important roles in the host–environment chemical interaction.

Petrosia ficiformis (Poiret, 1789, Demospongiae, Petrosiidae) is a common Mediterranean sponge living on hard substrata between 5 and 45 m of depth. Its colour, mainly due to symbiotic Cyanobacteria, ranges from violet to brown according to the illumination rates of the environment. *P. ficiformis* hosts a variety of heterotrophic bacteria, most of which live, together with Cyanobacteria, within specialised cells called bacteriocytes (Vacelet and Donadey, 1977). Genus *Petrosia* has been recognised as a source of diverse metabolites (Kim et al., 1998; Lim et al., 2001). Recently, a natural marine product (Polyacetylenetriol), from the Mediterranean sponge *Petrosia* sp., was found to be a novel general potent inhibitor of DNA polymerases (Loya et al., 2002).

It is widely accepted that culture-based techniques are inadequate for studying bacterial diversity from environmental samples, as many bacteria cannot be cultured and/or adequately identified using current and traditional techniques (Amann et al., 1995). The use of molecular approaches to describe microbial diversity has greatly enhanced the knowledge about natural microbial communities (Friedrich et al., 1999, 2001; Holben and Harris, 1995; Webster et al., 2001; Hentschel et al., 2002). However, microbial cultivation is essential to search for new bioactive compound producing strains.

The present study is aimed at investigating the diversity of epibiotic bacteria from a Ligurian population of *P. ficiformis*, focusing on the cultivable heterotrophic fraction of the surface microbial community. Epibacterial diversity was evaluated with both bacteriological and molecular approaches, and a screening of epibiotic bacteria able to produce antibacterial metabolites was performed, whose identification was assessed by partial 16S rRNA gene sequencing.

2. Materials and methods

2.1. Collection, isolation and phenotypic characterisation of the culturable epibiotic bacteria

Six sponge samples of *P. ficiformis* (arbitrarily numbered A, B, D, E, F and G) were collected at Paraggi (Portofino Promontory, Ligurian Sea) at a depth of 8 m. Samples were cut underwater using sterile scalpels, and taken to the laboratory in sterile containers at temperature of 20 °C. Prior to analysis, each sponge specimen was rinsed in sterile seawater. Viable heterotrophic bacteria were obtained swabbing a small area (ca. 1 cm^2, in three replicates) of each specimen's external surface with a sterile cotton-swab, which were then placed in 2 ml of sterile seawater, and vortexed. Serial 10-fold dilutions of each solution were prepared and aliquots (0.1 ml) were plated on Marine Agar 2216 (MA, Difco), and Thiosulphate Citrate Bile Sucrose (TCBS, Difco) Agar, in replicates. Plates were incubated for 7–10 days at 20 °C. Colony-forming units (CFU) were counted and the average number of bacteria referred to the swabbed area (cm^2). Perceptible different morphotypes were isolated in pure culture on MA. Bacterial isolates were kept in slant cultures at 4 °C.

Purified strains were characterised using morphological and biochemical tests according to standard methods: catalase test (3% H_2O_2), oxidase test (Kovacs, 1956), glucose, sucrose and lactose assimilation (1% w/v) (Hansen and Sorheim, 1991). Cell morphology, Gram stain and motility were observed using light microscopy. Proteinase activity was tested by using Skim Milk Agar plates (Peptone 10 g l^{-1}, Yeast extract 5 g l^{-1}, NaCl 10 g l^{-1}, sterile skim milk 10%) (Collins and Lyne, 1995). Isolates were tentatively identified to either the genus or the species level by comparing their phenotypic characteristics and biochemical test results to typed marine strains. Epibacterial diversity was evaluated through cluster groups composition.

2.2. Randomly Amplified Polymorphic DNA (RAPD)

RAPD was performed on selected pure strains: a single colony of each strain was picked, suspended in deionised water and boiled for 5 min. PCR amplification was performed in 30 μl of final volume reaction mixture containing 2 μl of DNA template, 10 μl of RedTaq Mix (Sigma) and 3 pmol μl^{-1} of both forward primer and RAPD primer mix (Bando et al., 1998). PCR parameters were carried out in a Thermal Cycler (Biometra, Lit.) as follows: pre-denaturation (94 °C, 1 min), followed by 50 cycles of denaturation (94 °C, 10 s), annealing (45 °C, 20 s) and elongation (68 °C, 1 min); post-elongation was at 68 °C for 2 min. RAPD fragments were separated according to their sizes by 1.2% agarose gel electrophoresis in 1 × TAE buffer. Gels were stained with ethidium bromide and photographed under UV light. RAPD patterns were compared using ImageJ Software (Rasband, 2002) to estimate the relatedness of DNA samples. A dendrogram was constructed from the provided distance matrix calculated from the presence/absence of shared bands using the Fitch–Margoliash and Least Square Distance Methods (Fitch and Margoliash, 1967; Felsenstein, 1993). Standard strains (*Burkholderia cepacea* LMG1222, *Pseudomonas elongata* LMG2182 and *Vibrio parahaemolyticus* LMG4424) were used as the outgroup in each analysis.

2.3. Antimicrobial assay

Bacterial isolates were preliminarily screened in order to test their antimicrobial activity, using the spot-on lawn method on Mueller–Hinton Agar (MHA, Microbiol) plates as described by Mayr-Harting et al. (1972). Metabolite production was tested against the indicator strains of human or marine origin (Table 3). Drops of overnight culture of each tested strain were spotted onto agar plates seeded with active growing cells of the indicator organism. After incubation (24 h at 25 °C), the antimicrobial activity was determined as a clear zone of inhibition around the spots. Isolates showing ability to produce antibacterial compounds were chosen for further analysis.

2.4. Strain identification with 16S rDNA analysis

Two out of the five strains showing antibacterial activity were selected for the molecular identification by 16S rDNA partial sequencing. PCR amplification of 16S rRNA gene was performed in a total volume of 30 μl containing the appropriate reaction buffer and reagents, 1 U of Red Taq polymerase (Sigma) and the universal eubacterial primers (f27: 5′-TAAGCTTAGAGTTTGATCATGGCTCAG-3′ and r1385: 5′-TAAGCTTTACCTTGTTACGACTTCAC-3′). PCR conditions were performed in a Perkin Elmer Cetus 480 Thermal Cycler as follows: initial denaturation (94 °C, 2 min), 5 cycles of denaturation (94 °C, 1 min), annealing (45 °C, 1 min) and extension (72 °C, 2 min), followed by 25 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min).

PCR products conforming to expected molecular weights were cloned into pGEM-Teasy vector (Promega) and transformed into *Escherichia coli* XL1B. Recombinant plasmids were extracted and purified by Qiagen columns kit (Qiagen) and sequenced on a ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) using the Sp6 and T7 sequencing primers and the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem). The obtained partial 16S rDNA sequences were aligned using the ABI Prism software (Perkin Elmer) and analysed with the queries generated by BLAST of GenBank Database (Altschul et al., 1990).

3. Results

3.1. Enumeration, composition and diversity of the culturable epibiotic bacterial community

Viable counts of epibacteria in the six sponge specimens were performed on MA and TCBS media, as summarised in Table 1. The average of total bacterial counts on MA ranged from 2.9×10^3 to 1.3×10^4 CFU cm^{-2}. Viable counts on TCBS, *Vibrio* selective medium, were homogeneously distributed ranging from 1.4×10^3 to 3.2×10^3 CFU cm^{-2}.

A total of 57 aerobic heterotrophic bacterial strains were isolated, 58% of which were Gram-negative. Most of the bacterial isolates were pigmented (yellow, orange, red or brownish), catalase positive and oxidase negative (53%); 33% of strains were motile and

Table 1
Viable count of total culturable epibiotic bacteria isolated from *P. ficiformis* specimens

Sponge samples	Average bacterial density (CFU cm^{-2})	
	Marine Agar	TCBS Agar
A	3.6×10^3	1.5×10^3
B	5.4×10^3	2.2×10^3
D	9.5×10^3	3.2×10^3
E	7.8×10^3	2×10^3
F	1.3×10^4	2.4×10^3
G	2.9×10^3	1.4×10^3

14% produced extracellular proteases. Gram-positive strains were usually rod in shape, sometimes showing cell inclusions similar to spore formations, and organised in chains. Gram-negative strains were long or short rods and not spore-forming.

Carbohydrate assimilation tests revealed that most of the isolates could utilise glucose (70%) or sucrose (37%) as carbon source, whereas only 3% of strains were lactose fermenting. An equal distribution of strains bearing fermentative metabolism (46%) and oxidative metabolism (49%) was observed.

Isolates were assigned to 10 bacterial groups according to a presumptive identification by morphological and biochemical features (Table 2). Gram-negative bacteria were diversified in non-fermentative/oxidase positive (*Flavobacter*), oxidative (*Pseudoalteromonas*), fermentative/oxidase positive (*Vibrio* and *Aeromonas*) and fermentative/oxidase negative (Enterobacteriaceae) strains. Gram-positive bacteria were discriminated as spore-forming rods (*Bacillus*), not spore-forming rods (Corynebacteria), cocci (*Micrococcus*) and filamentous-branching rods (*Actinomyces* and *Streptomyces*). Other unidentified non-fermentative Gram-negative rods were placed in a separate group (Others).

Pseudoalteromonas, *Vibrio* and Corynebacteria were consistently recovered and had homogeneous distribution in all specimens, while *Actinomyces*, *Streptomyces*, Enterobacteriaceae and *Aeromonas* were occasionally isolated.

Nineteen Gram-negative non-fermentative isolates and 10 not spore-forming Gram-positive strains were randomly chosen from different sponge samples to assess their genetic similarity using RAPD fingerprinting technique (Fig. 1). A total of 24 distinct RAPD profiles were assessed yielding 2–14 bands between 2000 and 100 bp.

Fig. 2 shows the maximum-likelihood trees based on the provided distance matrix obtained from the alignment of RAPD profiles within not spore-forming Gram-positive bacteria (Fig. 2a) and Gram-negative non-fermentative bacteria (Fig. 2b). Reference strains (*B. cepacea* LMG1222, *P. elongata* LMG2182 and *V. parahaemolyticus* LMG4424) were used as the outgroup in each of these analyses.

The 10 not spore-forming Gram-positive strains analysed (Fig. 2a) yielded eight RAPD profiles. RAPD profiles of sponge clones E1 and E2 were very similar but not identical to that of the isolates A10 and A13 (clones) (cluster P1). Five strains showed few genetic differences, indicating that they form a homogenous group (cluster P3), whereas strain E4 appeared related to the reference strains.

The tree representing genetic relatedness among the 19 Gram-negative isolates was composed of four clusters. Cluster C2 included two multiple isolates from the same

Table 2
Microbial composition from *P. ficiformis* sponge samples

Sponge sample	Number of isolates										
	Pseudoalteromonas	*Flavobacter*	*Micrococcus*	*Bacillus*	Corynebacteria	*Actinomyces*, *Streptomyces*	*Vibrio*	*Aeromonas*	Enterobacteriaceae	Other	Total number
A	6	2	0	2	2	0	2	0	0	2	16
B	1	0	2	2	0	0	1	0	0	0	6
D	4	0	2	0	1	0	1	0	0	0	8
E	1	1	0	0	8	0	1	0	1	0	12
F	2	1	1	1	0	0	1	1	0	2	9
G	1	0	0	0	2	1	1	0	0	1	6

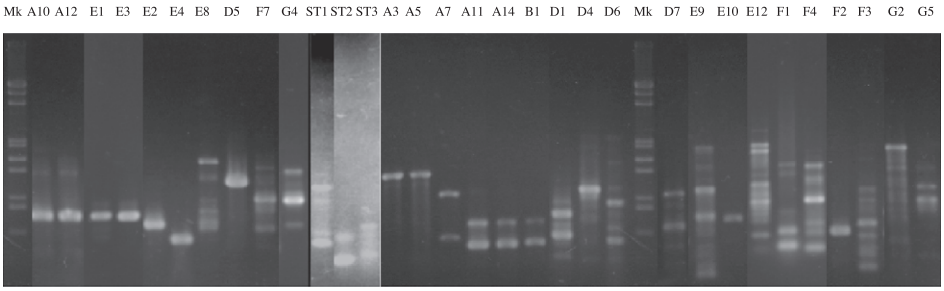

Fig. 1. Diversity of marine bacteria determined by RAPD fingerprinting of sponge isolates. Mk = *Lambda Hin*d/*Eco* digests; lanes A10–G4 = Corynebacteria; lanes F3, E12, A7 = *Flavobacter*; lane F1 = *Aeromonas*; lanes A3, A5, A11–D6, D7, E10, F4–G5 = *Pseudoalteromonas*; ST1 = *V. parahaemolyticus* LMG4424; ST2 = *P. elongata* LMG2182; ST3 = *B. cepacea* LMG1222.

sponge specimens (A3 and A5), displaying similar patterns to strain F1 and *V. parahaemolyticus* reference strain. Strains with identical profiles were also isolated from different sponges (A11, A14 and B1). Cluster C1 showed high homogeneity and relation with *B. cepacea*, whereas strains F3 and E12 branched far from any cluster and could be allocated separately.

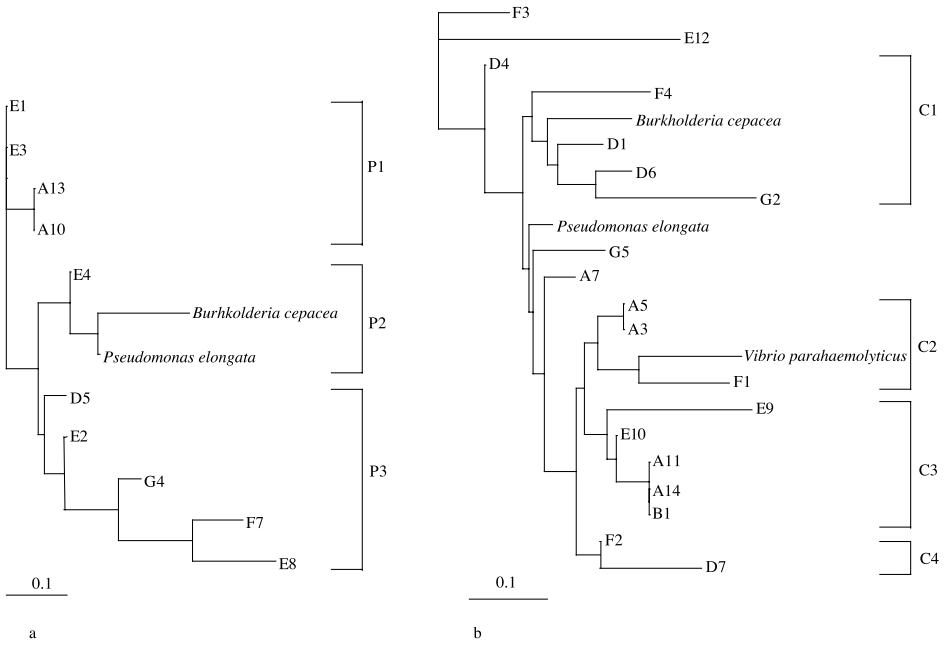

Fig. 2. Cluster analysis using Fitch–Margoliash method to measure the similarity among the Corynebacteria isolates (a) and among the Gram-negative isolates belonging to *Pseudoalteromonas*, *Aeromonas* and *Flavobacter* genera (b). Scale bar represents 10 mutations per 100 nucleotide positions. A, B, D, E, F, G = sponge samples; P, C = clusters; reference strains: *V. parahaemolyticus* LMG4424; *P. elongata* LMG2182; *B. cepacea* LMG1222.

Table 3
Screening for antimicrobial activities against marine and human reference strains

Strain	Presumptive identification	Indicator lawns							
		E. faecalis	*S. aureus*	*Micrococcus* sp.	E2	E3	E7	E8	F3
D5	*Corynebacterium*	–	–	+	–	–	–	–	–
F6	*Pseudoalteromonas*	+	–	–	–	–	–	–	–
E1	*Rhodococcus*	–	+	+	+	+	–	+	–
E9	*Pseudoalteromonas*	–	–	–	+	+	+	+	–
E12	*Pseudoalteromonas*	–	–	–	–	–	+	–	+

+ = inhibition zones around spot; – = no activity; D–E = bacterial isolates.

3.2. Antibacterial activity

Bacterial isolates from *P. ficiformis* were assayed for their antimicrobial activity against both human pathogens and sponge isolates. Five strains induced inhibition of one or more indicator strains (Table 3). Two strains obtained from the same sponge specimen (E1 and E9) produced antibiotic compounds active against *Staphylococcus aureus* and other Gram-positive rods and were chosen for DNA sequencing.

Strain E1 formed slow-growing, small-sized, orange-pigmented colonies. It had a Gram-positive irregular rod shape and was catalase positive, oxidase negative and not motile. The 16S rDNA partial sequence analysis (1025 bp) showed that it was closely related to the Actinomycetales group, with a 98% sequence similarity to *Rhodococcus erythropolis* (Genbank accession no. AY147846).

Strain E9 was characterised as a Gram-negative motile rod, catalase positive and oxidase negative. The 16S rDNA partial sequence analysis (711 bp) showed a high percentage of similarity (98%) to the 16S rDNA partial sequences of strains belonging to the genus *Pseudomonas* (Genbank accession no. AF326356 and AF388209).

4. Discussion

The total bacterial densities recorded from the surface of the sponge *P. ficiformis* indicate slight differences in the numbers of viable bacteria isolated from different specimens. On TCBS selective medium, viable counts were roughly equivalent from every sponge sample. Literature data reporting microbial densities for sponges only refer to the mesohyl communities, since study has rarely addressed the problem of epibiotic microbial flora (Becerro et al., 1994). Nevertheless, epibacterial densities reported for other marine invertebrates such as ascidians, bryozoans and several aquatic plants (Wahl, 1995; Santavy and Colwell, 1990), appear to be higher than those observed on *P. ficiformis*, which could suggest that the surface of this sponge bears some devices to regulate microbial epibiosis.

Conforming to data presented by other authors (Vacelet and Donadey, 1977; Wilkinson, 1978; Sochard et al., 1979; Santavy and Colwell, 1990; Ward-Rainey et al., 1996) *Pseudoalteromonas*, *Flavobacter* and *Vibrio* were common invertebrate-associated bacterial genera. Among the other genera isolated, Corynebacteria, *Bacillus* and *Micrococcus*

were numerically well represented. Enterobacteriaceae, *Aeromonas*, *Actinomyces* and *Streptomyces* were recovered rarely.

Pseudoalteromonas is a common genus widely retrieved from marine microfouling communities associated to eukaryotic hosts; it is known to produce many bioactive compounds which help to compete for nutrients and for the colonization of surfaces (Holmström and Kjelleberg, 1999; De Rosa et al., 2000; Egan et al., 2001). Many substances derived from *Pseudalteromonas* species can either inhibit fouling or facilitate settling of other organisms (Holmström and Kjelleberg, 1999), therefore this genus has a strong potential for community structuring. *Pseudalteromonas* species have also often been related to marine invertebrate diseases (Holmström and Kjelleberg, 1999).

Corynebacteria, *Actinomyces* and *Streptomyces* are widely distributed in the marine environment. They are considered a source of bioactive agents and display competitive biosynthetic capabilities. Several isolates, associated to marine plants and animals, have been recently reported to express antibacterial, antifungal and antitumor activities (Zheng et al., 2000).

In the marine environment, *Bacillus* species are usually isolated from mud and sediments, but they are sometimes found associated with marine benthos (Hentschel et al., 2001). As most of other *Bacillus* species, marine *Bacillus* are able to produce peptide compounds with antimicrobial activity (Barsby et al., 2001; Hentschel et al., 2001; Jaruchoktaweechai et al., 2000).

Micrococcus are widely spread in the marine environment; they are usually free-living bacteria dispersed in the water column, but may occasionally be found attached to either living or non-living submerged surfaces. Recently, Gram-positive bacteria belonging to genus *Micrococcus* were collected from the mesohyl of two sponges and new bioactive compounds were isolated and analysed for their biological activity (Stierle et al., 1988).

Bacteria of the genus *Vibrio* are typically found in aquatic habitats where they are often components of microfouling. Several species are pathogens for fishes, whereas luminous *Vibrio* are true symbionts for squids and nudibranchs. In some cases, *Vibrio* species were found involved in coral and shellfish diseases (Israely et al., 2001). *Vibrio* strains associated with the sponge *Hyatella* sp. were found to produce a peptide antibiotic (Oclarit et al., 1994).

Motile bacteria belonging to the genus *Aeromonas* are often ubiquitous members of the water ecosystem. *Aeromonas* strains can also be components of the microbial flora of aquatic animals and may be pathogens of fishes, crustaceans and even humans (Starliper and Morrison, 2000).

Enterobacteriaceae are a very large family of some 20 genera of facultatively anaerobic Gram-negative rods. Enterobacteria are widely disseminated in soil, water, plants and animals. Some can be opportunistic pathogens of fishes, reptiles and insects as well as of mammals, birds and plants. Enterobacteriaceae, and especially *Escherichia coli*, are considered to be indicators of faecal contamination (Baudisova et al., 1997).

Marine *Flavobacter* are ubiquitous, and are estimated to occur from non-detectable levels to more than 40% of the viable flora (Jooste and Hugo, 1999). Most *Flavobacter* are highly proteolytic and are known to produce extracellular lipases (Labuschagne et al., 1997).

RAPD analysis is commonly used to differentiate closely related strains of bacteria and recent works applied RAPD technique to evaluate differences among total bacterial aquatic communities (Franklin et al., 1999; Uphoff et al., 2001). Molecular fingerprinting analysis of both non-fermentative Gram-negative and not spore-forming Gram-positive bacteria from the surface of *P. ficiformis* showed the presence of genetically related strains within each group. Genetic clones existed prevalently as multiple isolates from the same sponge sample.

Both RAPD analysis and classical phenotypic methods show that the surface of the marine sponge *P. ficiformis* harbours a relatively restricted range of microorganisms. The low epibacterial densities, summed to a substantially constant microbial array, could suggest that the microflora of *P. ficiformis* follows a defined pattern and that some homeostatic mechanisms may be present to maintain the balance. These mechanisms are still unclear and only some hypotheses can be proposed:

(i) *P. ficiformis* could produce antimicrofoulants to prevent dense microbial aggregations on its surface; sponges are known to produce bioactive compounds whose antifouling properties are being studied (De Rosa et al., 2000);
(ii) since numerical dominances by *Pseudoalteromonas* and *Vibrio* species were detected in all of the studied microbial communities, these strains could limit or inhibit the development of other bacteria therefore keeping microbial densities at a low and constant level. This has already been postulated for *Pseudalteromonas* (Holmström and Kjelleberg, 1999). Moreover, in complex microbial communities, direct competition can induce antibiotic production even in strains that usually do not release bioactive metabolites (Burgess et al., 1999);
(iii) both of these factors could act synergistically or could be related. No study, at the moment, has clarified whether bioactive metabolites isolated by *Petrosia* sp. are produced by the sponge itself or by an associated microorganism. This possibility has already been demonstrated for other sponges known to produce active compounds (Stierle et al., 1988; De Rosa et al., 2000; Hentschel et al., 2001) proving that *Micrococcus* and *Pseudoalteromonas* species were often involved. Therefore, *P. ficiformis*-associated strains could account for its antifouling properties and play a role in the development and structuring of its superficial microbial community. This strategy would allow the sponge to reduce energy costs for defence against more harmful fouling organisms (Wahl, 1989).

In this study, five epibiotic bacteria retrieved from *P. ficiformis* were able to inhibit in vitro the bacterial growth of other marine strains which were also isolated from sponges. These strains could play a role in the microbial community dynamics of the external microflora of *P. ficiformis* limiting its densities. Their role could be ecologically important in defining temporal succession patterns or bacterial success within the community. Two isolates showed a wide range of antibacterial activity. The molecular identification by partial 16S rRNA gene sequencing revealed that they were closely related to the genera *Rhodococcus* and *Pseudomonas*. It has recently been demonstrated that sponge isolates with antimicrobial activity are numerically very abundant in the genus *Pseudoalteromonas* and the group of α-Proteobacteria (Hentschel et al., 2001; Olson et al., 2002). Also,

Actinomycetes recognised as sources of antimicrobial compounds have been isolated from various marine invertebrates, such as corals and sponges (Zheng et al., 2000).

The high recovery of strains with antimicrobial activity suggests that marine sponges represent an ecological niche which harbours a largely uncharacterised microbial variety and a yet unexploited potential in the search for new secondary metabolites.

As previously demonstrated in other works (Kim et al., 1998; Lim et al., 2001; Loya et al., 2002), various substances extracted from *Petrosia* sp. inhibit prokaryotic and eukaryotic cell growth. Several applications such as the development of new antifouling compounds as well as novel antimicrobial agents to be employed in research and medical fields are major targets of current interest.

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