

Antifouling Activities against Colonizer Marine Bacteria of Extracts from Marine Invertebrates Collected in the Colombian Caribbean Sea and on the Brazilian Coast (Santa Catarina)

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Z. Naturforsch. **66c**, 515–526 (2011); received December 10, 2010/June 28, 2011

The growth inhibition of 12 native marine bacteria isolated from *Aplysina* sponge surfaces, the shell of a bivalve, and PhytigelTM immersed for 48 h in sea water were used as indicator of the antifouling activity of the extracts of 39 marine organisms (octocorals, sponges, algae, and zoanthid) collected in the Colombian Caribbean Sea and on the Brazilian coast (Santa Catarina). Gram-negative bacteria represented 75% of the isolates; identified strains belonged to *Oceanobacillus iheyensis*, *Ochrobactrum pseudogrignonense*, *Vibrio campbellii*, *Vibrio harveyi*, and *Bacillus megaterium* species and seven strains were classified at genus level by the 16S rRNA sequencing method. The extracts of the octocorals *Pseudopterogorgia elisabethae*, four *Eunicea* octocorals, and the sponges *Topsentia ophiraphidites*, *Agelas citrina*, *Neopetrosia carbonaria*, *Monanchora arbuscula*, *Cliona tenuis*, *Iotrochota imminuta*, and *Ptilocaulis walpersii* were the most active, thus suggesting those species as antifoulant producers. This is the first study of natural antifoulants from marine organisms collected on the Colombian and Brazilian coasts.

Key words: Antifouling Activity, Antibacterial Activity, Marine Invertebrate-Associated Bacteria, 16S rRNA Gene Analysis

Introduction

Fouling organisms, mainly consisting of microbial slimes, diatoms, barnacles, tunicates, bryozoans, and spores of algae, cause extensive damage to commercial important man-made structures exposed to sea water, by forming a complex layer on their surfaces as a result of adhesion of marine organisms (Fusetani, 2004). This phenomenon known as biofouling is viewed as a serious economic problem estimated to be about \$ 5 billion/year considering only the shipping industry (Fusetani and Clare, 2006). For many years, different antifouling technologies have been developed with the result that the only solution of the problem is to cover exposed surfaces with paints based on copper or tin compounds, which kill the fouling organisms by a continuous release of organometallic compounds to the surrounding sea water (Yebra *et al.*, 2004). However, the adverse effect of those organometallic compounds, such

as toxicity and other harmful effects (*i.e.* imposex) on non-target organisms, is well recognized up to a point where the use of these paints has been banned in many countries (Fusetani and Clare, 2006). Recently, as an alternative to toxic compounds, the use of natural products present in marine organisms, which might be toxic but biodegradable, turns into an interesting option to incorporate them into environmentally friendly antifouling paints. Those antifouling compounds could be isolated from sessile marine animals free from fouling which produce such compounds presumably as a means of protection from predation or fouling or to reduce competition for space. Some of the studied organisms are sponges, algae, and bryozoans, in which terpenoid isocyanate compounds, 3-alkylpyridinium compounds, brominated furanones, and brominated indole alkaloids have been found as secondary metabolites which can control the colonization of fouling organisms (Fusetani, 2004, 2011).

The first step in the development of natural products as commercial antifoulants is the evaluation of the antifouling potential of the source. A broad selection of marine organisms has been used as a model for antifouling bioassays including barnacles, macroalgae, bivalves, marine fungi, and bacteria, attempting to cover a wide selection of the most common colonizer organisms in different geographical conditions (Briand, 2009). It has been established that bacteria are the first organisms to colonize immersed surfaces (Krug, 2006). They associate with soft-bodied organisms or with inert surfaces and form biofilms which have long been recognized as fundamental settlement cues (positive or negative stimuli) for the attachment and growth of other organisms such as other bacteria, invertebrate larvae or spores of algae (Dobretsov and Qian, 2004). Thus, the bacterial community is often involved in the process of fouling by regulating the settlement of organisms and by promoting or inhibiting colonization (Dobretsov and Qian, 2004; Qian *et al.*, 2007; Briand, 2009).

According to the above mentioned considerations and as part of our continuous search for useful compounds from marine organisms collected in the Colombian Caribbean Sea (Duque *et al.*, 2006; Tello *et al.*, 2009; Correa *et al.*, 2009) and on the Brazilian coast (Lhullier *et al.*, 2010), we isolated and identified 12 marine bacterial strains associated with immersed fouled surfaces and used them to test the antibacterial activity of 39 extracts from clean surfaces of marine organisms as a first approach to evaluate the presence of antifouling compounds in these extracts.

Methods

Isolation of marine surface colonizer bacteria

Marine bacteria were obtained from the external pinacoderm and from the grooves formed by the intake pores (ostium) of *Aplysina lacunosa* pinacoderm (strains 3–7 and 12), from the external pinacoderm of *Aplysina insularis* (strains 8 and 9), from the calcareous surface of the abandoned shell of *Donax* sp. (strains 1 and 2), and from Phytigel™ immersed in sea water for 48 h (strains 10 and 11). The organisms were collected using scuba diving at depths of 5–15 m at the Granate Ensenada (Tayrona Natural National Park, North of the Colombian Caribbean). The bacteria were

isolated from the sponges as follows. Surface fragments of the sponges were cut underwater (approx. 1 cm² each), using gloves and sterile scalpels, and taken immediately to the laboratory in sterile Whirl-Pack® bags. Each sponge sample was then rinsed three times in sterile sea water. Bacteria were obtained swabbing the external surface of each fragment with a sterile cotton swab and were then placed in 5 mL of sterile sea water and vortexed. Serial 6-fold dilutions of each solution were prepared and aliquots (0.1 mL) were plated on glycerol artificial seawater medium (GASW) (Smith and Hayasaka, 1982; Mora-Cristancho *et al.*, 2008) in two replicates. Plates were incubated for 8 d at room temperature [(25 ± 3) °C]. Isolation of bacteria from Phytigel™ gels was performed as follows. Phytigel™ (0.53 g) was added to 50 mL distilled water. The gel mixture was heated until boiling and poured into 10-cm diameter Petri plates following the method of Henrikson and Pawlik (1995). Four Petri plates were placed 2 m below the water surface in Santa Marta Bay for 48 h, and the bacteria were obtained by swabbing the surface of these gels.

All cultured isolates were selected by colony morphological characteristics and purified by repeated single colony subculture in the same media. These were also kept in slant cultures at 4 °C and at room temperature.

Identification of bacterial strains

DNA extraction and 16S rDNA sequencing

DNA was extracted using standard phenol/chloroform extraction procedures. Polymerase chain reaction (PCR) amplification was performed in a total volume of 50 µL containing 1X PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl], 200 µM of each deoxynucleotide, 1.5 mM MgCl₂, 2.5 U Taq DNA polymerase (Invitrogen TM, Carlsbad, CA, USA), 50 ng DNA, and the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane, 1991). PCR conditions were as follows: initial denaturation for 2 min at 95 °C, followed by 30 cycles of denaturation for 1 min at 95 °C, primer annealing for 1 min at 56 °C, and primer extension for 1.3 min at 72 °C with a cycle of final extension for 10 min at 72 °C. The PCR amplification product was purified and sequenced using the sequencing services of Macrogen (Seoul, Korea).

Sequence analyses

Each sequence was defined as an operational taxonomic unit (OTU). Sequences were assembled in the CAP3 DNA sequence assembly program (Huang and Madan, 1999) and subsequently aligned using the software Clustal X v.2.0.10 (Larkin *et al.*, 2007). A dendrogram was constructed using the neighbour-joining algorithm (Saitou and Nei, 1987) and Kimura two-parameter method (Kimura, 1980) and processed using MEGA software (Tamura *et al.*, 2007). The robustness of the inferred tree topology was evaluated with 1000 bootstrap replicates.

Nucleotide sequence accession numbers

The GenBank accession numbers for the sequences generated in this study are GQ203107 through GQ203118.

Evaluation of antifouling activity of marine invertebrate extracts

Preparation of extracts

The 33 marine invertebrates were collected at the Santa Marta Bay and at the Rosario Islands, Colombia, by means of scuba diving, between September 2005 and March 2006. The samples included the soft corals *Eunicea knighti*, *Eunicea succinea*, *Eunicea fusca*, *Eunicea* sp. 2, *Eunicea tayrona*, *Eunicea laciniata*, *Muriceopsis* sp., *Erythropodium caribaeorum* and two chemotypes of *Pseudopterogorgia elisabethae* (chemotype 1 and chemotype 2); the sponges *Biemna cribaria*, *Iotrochota imminuta*, *Dragmacidon reticulata*, *Oceania peltata*, *Cinachyrella kuekenthali*, *Spirastrella coccinea*, *Cliona tenuis*, *Cliona delitrix*, *Agelas conifera*, *Agelas citrina*, *Monanchora arbuscula*, *Svenzea tubulosa*, *Neopetrosia proxima*, *Neopetrosia subtriangularis*, *Neopetrosia carbonaria*, *Xestospongia muta*, *Neopetrosia rosariensis*, *Petrosia pallasarca*, *Ptilocaulis wallpersi*, *Topsentia ophiraphidites*, *Lissodendoryx colombiensis*, *Amphimendon caycedoy* and the zoanthid *Palythoa caribbaeorum*. Identification of the species was done by Dr. Sven Zea and Dr. Mónica Puyana. Vouchers of each one of the studied species are at the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, Bogotá, Colombia and at the Instituto de Investigaciones Marinas y Costeras “José Benito Vives De Andrés” Invemar, Santa Marta, Colombia Collections. Once the organisms had been collected and rinsed with

sterile sea water to remove associated debris, they were frozen until extraction. Then the samples were cut into small pieces and extracted by maceration in CH₂Cl₂/MeOH (1:1) for 24 h, three times. The extract, obtained after filtration, was then concentrated to dryness under vacuum at 40 °C, and stored at 4 °C until use.

The algae were collected at Santa Catarina coast, by means of scuba diving, in September 2005, March 2006, and February 2008. The samples included the five *Laurencia* species: *L. catarinensis*, *L. microcladia*, *L. majuscula*, *L. flagelifera*, and *L. obtusa*, and one *Peyssonelia* species: *P. capensis*. Identification of the species was done by Prof. Paulo Antunes Horta Junior, Department of Botany, Federal University of Santa Catarina, Florianopolis, Brazil. Vouchers of each one of the studied species have been deposited at the herbarium of this department. After collection and rinsing with sterile sea water to remove associated debris, the algae were air-dried. Then the samples were cut into small pieces and extracted by maceration in MeOH, CH₂Cl₂/MeOH (1:1), and CH₂Cl₂ for 48 h each. The extract, obtained after filtration, was concentrated to dryness under vacuum at 40 °C, and stored at 4 °C until use.

Antibacterial activities

The test of the extracts against the Caribbean marine bacteria isolates was performed using a standard agar disc diffusion assay (Hewitt and Vincent, 1989). Bacterial strains were incubated between 18 and 24 h in artificial marine broth (Smith and Hayasaka, 1982; Mora-Cristancho *et al.*, 2008) at (25 ± 3) °C. Bacteria concentration was adjusted to 10⁸ CFU mL⁻¹ with 0.5 McFarland standard before spreading the bacterial suspension onto GASW agar. Whatman GF-C filter paper discs (5.2 mm i.d.) were loaded with 300 µg of extract and then placed in the agar with the bacteria. After incubation for 48 h at (25 ± 2) °C, the diameter of the inhibition zones around the discs was measured. Control tests with the solvents CH₂Cl₂, MeOH, and DMSO were carried out in each plate showing no inhibition of bacterial growth. In addition, positive control discs with 30 µg of the biocide Cu₂O (10 µg mL⁻¹ in water) were used. All inhibition assays were carried out in triplicate. The data presented here corresponds to the average of the diameter of inhibition zone.

Results and Discussion

Molecular characterization of marine bacteria used for standard bioassays

Twelve bacterial isolates from surfaces of the sponges *Aplysina insularis* and *Aplysina lacunosa*, from the shell of the bivalve *Donax* sp., and from Phytigel™ Petri plates immersed for 48 h in sea water were isolated, characterized by biochemical tests, macro- and microscopically, and identified by 16S rRNA gene sequences analyses (Table I).

All characterized isolates were Gram-negative except 1, 4, and 9. They grew aerobically at room temperature. Standard biochemical tests were performed for preliminary identification [citrate, lysine decarboxylase, sulfide-indol-motility (SIM), and methyl red (MR), data not shown].

The 16S rRNA genes of the marine bacterial isolates were sequenced and the sequences subsequently aligned to currently available 16S rRNA gene sequences; they showed significant similarity to sequences from the GenBank and the European Molecular Biology Laboratory (EMBL) nucleotide sequence databases. For comparison we selected the most similar sequences from recognized type strain collections such as ATCC (American Type Culture Collection). A derived multiple sequence alignment was used to generate a pairwise similarity matrix and genetic distances between our strains and related bacteria. Some of the sequences could be assigned to a bacterial species but others were sufficiently similar to the type strains (Table I).

The tree topology shown in Fig. 1 was obtained using the neighbour-joining method (Saitou and Nei, 1987), and distances were calculated with the Kimura two-parameter method computing the units of the number of base substitutions per site (Kimura, 1980). Statistical evaluation of genetic divergences was performed by bootstrap resampling of the sequence data.

Since Vacelet (1975), studies on sponge-associated microorganisms have focused on communities inhabiting the mesohyl and the interior of the sponge cells. Significant progress has been made in these studies. However, the knowledge about epibiotic bacteria and their role in the ecology of marine sponges is still unexplored. This study is one of the few in which bacteria have been isolated from sponge surfaces, and some of these bacteria correspond consistently to some of those reported in other studies on *Aplysina* spp. tis-

sue matrix (Friedrich *et al.*, 1999). Even though we found differences (probably due to the fact that we sampled the epibiotic community from the sponges) in general we can mention coincidences in case of some Gram-positive bacteria (*Bacillus*), γ -proteobacteria (*Vibrio*, *Pseudoalteromonas*), and α -proteobacteria (*Ochrobactrum*) (Friedrich *et al.*, 1999; Ahn *et al.*, 2003). Others of our isolates are similar to *Pseudoalteromonas*, *Vibrio*, and *Bacillus*, that are widespread bacteria in marine environments associated with sponges, tunicates, mussels, pufferfish, and algae (Rao *et al.*, 2005; Allers, 2007).

Strains 1, 3, 7, 9, and 11 had a satisfactory classification with identity percentages higher than 97%. Strain 1 has 98% similarity with *Oceanobacillus iheyensis* strain HTE831, which was isolated from deep sea mud and is extremely halophilic and alkaliphilic. These characteristics are common also for our isolate (Liu *et al.*, 2001). However, we found this bacterium in relatively shallow depth and as biofilms on calcareous surfaces. Strain 3 has 99% similarity with *Ochrobactrum pseudogrignonense*. It is interesting to note that such species has been isolated mostly in the East China Sea, and there are no reports on this species in the Caribbean Sea. It has been related with *N*-acyl-homoserine lactone degradation that could be a clue to its role on the surfaces of non-fouled organisms (Zhou *et al.*, 2008). Strain 7 has 97% sequence similarity with *Vibrio campbellii* which has been isolated several times from sea water. *V. campbellii* occurs in the open ocean at high frequency, and it has been proposed that it represents an autochthonous species in surface waters of the Atlantic Ocean (Grimes *et al.*, 1986). Strain 9 is the only one showing 100% similarity with a known species, *i.e.* *Bacillus megaterium*. Strain 11 has 98% similarity with *Vibrio harveyi*. This bacterium is common in the oceans of the world; it can be found easily in non-extreme environments and subtropical waters. It is thought that *Vibrio* are the most abundant cultivatable bacteria in marine environments. It has been reported that the genera *Vibrio*-*Photobacterium* usually represent up to $2 \cdot 10^8$ cells L^{-1} out of the $2.6 \cdot 10^{10}$ bacteria (Heidelberg *et al.*, 2002). They are present in normal microbiota of common marine organisms and are associated with both diseased and healthy aquatic animals (Thompson *et al.*, 2003).

The isolates identified in this study as *Ochrobactrum pseudogrignonense*, *Vibrio campbellii*,

Table I. Percentage of 16S rDNA sequence identity and evolutionary distances of marine bacterial strains compared with the most similar species found in data bases.

Strain No.	Strain source	Gram staining	Strain designation ^a	Accession No.	Best match (% of similarity) ^b	Kimura two-parameter distance ^c	Most similar species ^d
1	<i>Donax</i> sp.	+	31-C	GQ203116	<i>Oceanobacillus ihyensii</i> (98.1)	0.020	<i>Alteromonas macleodii</i>
2	<i>Donax</i> sp.	-	29-C	GQ203115	<i>Alteromonas macleodii</i> (95)	0.050	
3	<i>Aplysina lacunosa</i>	-	4-4DEP	GQ203110	<i>Ochrobactrum pseudogrignonense</i> (99.2)	0.008	
4	<i>Aplysina lacunosa</i>	+	10-4DEP	GQ203109	<i>Kocuria</i> sp. DQ448709.1 (99.9)	0.001	<i>Vibrio harveyi</i> ; <i>V. natriegens</i> ; <i>V. proteolyticus</i>
5	<i>Aplysina lacunosa</i>	-	11-6DEP	GQ203111	<i>Kocuria rosea</i> (96.5)	0.036	
					<i>Vibrio harveyi</i> (95.7)	0.044	
6	<i>Aplysina lacunosa</i>	-	21-6PIN	GQ203113	<i>Ochrobactrum pseudogrignonense</i> (96.3)	0.037	<i>Schewanella</i> sp.
7	<i>Aplysina lacunosa</i>	-	6-8PIN	GQ203112	<i>Vibrio campbellii</i> (99.6)	0.004	
8	<i>Aplysina insularis</i>	-	12-Aims6B	GQ203107	<i>Schewanella algae</i> (95.1)	0.050	
9	<i>Aplysina insularis</i>	+	16-Aims5	GQ203108	<i>Bacillus megaterium</i> (100)	0.000	<i>Vibrio parahaemolyticus</i> ; <i>V. proteolyticus</i> ; <i>V. alginolyticus</i>
10	Phytigel	-	Phy-2A	GQ203118	<i>Vibrio harveyi</i> (95.4)	0.047	
11	Phytigel	-	Phy-11	GQ203117	<i>Vibrio harveyi</i> (97.7)	0.023	
12	<i>Aplysina lacunosa</i>	-	23-6PIN	GQ203114	<i>Vibrio alginolyticus</i> (96.8)	0.033	<i>V. parahaemolyticus</i>

^a Strain designation in our strain collection: C, from a *Donax* sp. shell; DEP, the grooves formed by the intake pores (ostium) of *Aplysina lacunosa*; PIN, external pinacoderm of *Aplysina lacunosa*; Aims, external pinacoderm of *Aplysina insularis*; Phy, PhytigelTM immersed in sea water.

^b Estimate of divergence between sequences, calculated with the number of base differences per site. p distance is the proportion of nucleotide sites at which two sequences being compared are different).

^c Estimates of evolutionary divergence between sequences calculated with the number of base substitutions. Analyses were conducted using the Kimura two-parameter method (Kimura, 1980) The Kimura two-parameter distance takes into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites.

^d Most similar species found in the data bases EMBL and GenBank.

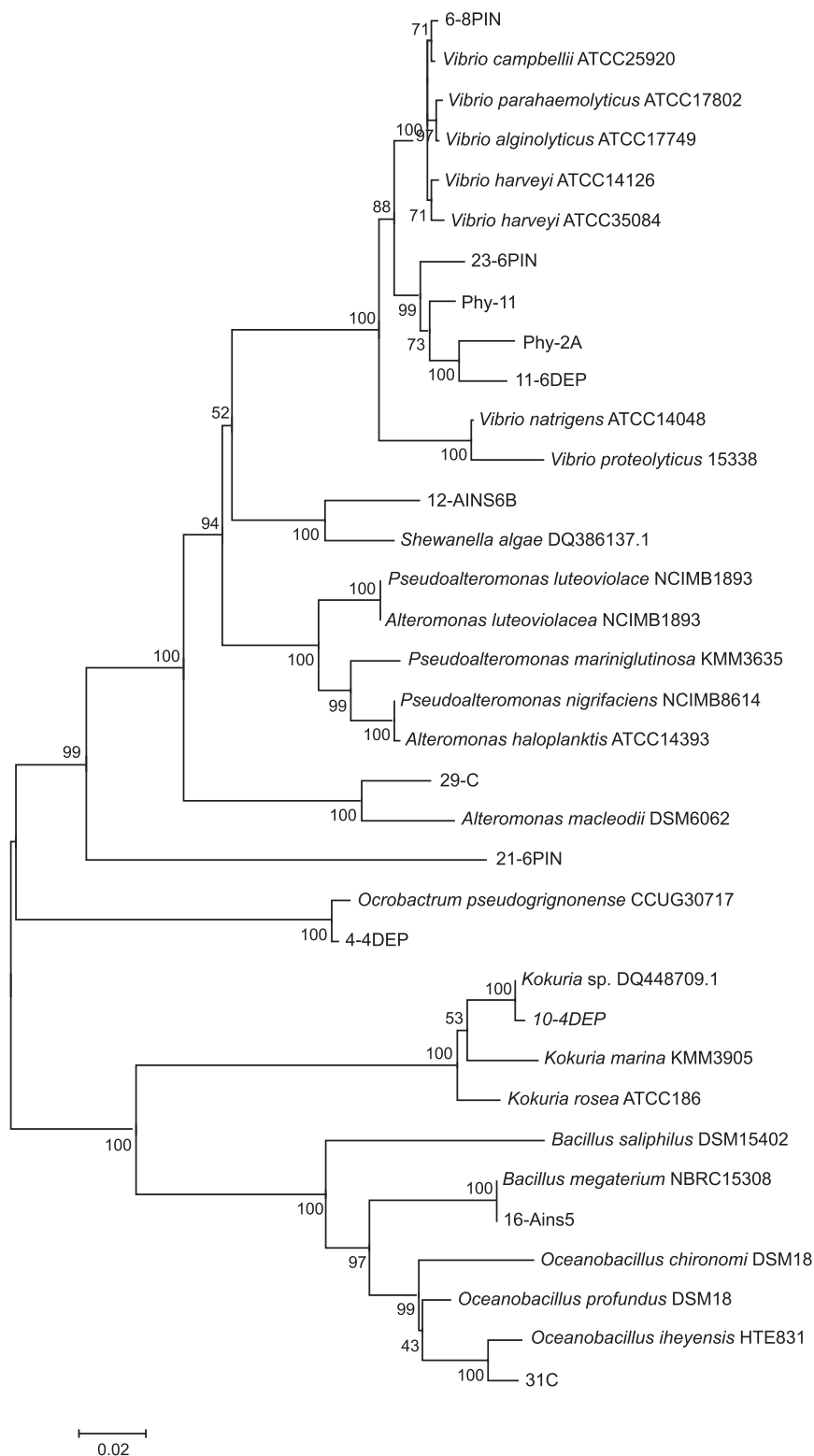


Fig. 1. Tree of marine bacterial isolates based on 16S rDNA gene sequence. The dendrogram was inferred using the neighbour-joining method. The optimal tree with the percentage of replicate trees, in which the associated taxa are clustered, and the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura two-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA4. Collections: ATCC, American Type Culture Collection; NCIMB, National Collection of Industrial, Marine and Food Bacteria; KMM, Collection of Marine Microorganisms of the Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM in figure); CCUG, Culture Collection of the University of Göteborg; NBRC, NITE Biological Resource Center, Japan; HTE831 is the type strain of the Japan Agency for Marine-Earth Science and Technology [Japan MSTC (JAMSTEC)]. PIN, Phy, Ains, C, and DEP refer to the designation in our strain collection (Table I).

and *Bacillus megaterium* have not been reported before for *Aplysina* sponges. Isolate 4 is related to high G+C Gram-positive bacteria (*Kocuria*) having 96.5% sequence similarity with *Kocuria rosea* and even 99.9% with another *Kocuria* strain that has not yet been properly classified (*Kocuria* sp. DQ448709.1) (Gontang *et al.*, 2007). It may be interesting to explore if our isolate and the referenced one could belong to a species taxonomically separated from that genus.

For strain 2, further comparison is required to find out to which species of the genus *Alteromonas* it is most similar, given that the present members of this taxon are related by 16S rDNA sequence identities below 95%. The same is true for strains 5, 6, 8, 10, and 12 which are between 95 and 97% similar to the most comparable species found in the data bases (Rosello-Mora and Amann, 2001).

Antibacterial activity of extracts of marine invertebrates

The antibacterial activity of extracts of 33 marine invertebrates (soft corals, sponges, and a zoanthid) collected in the Colombian Caribbean Sea and of 6 marine algae collected on the Brazilian coast against 12 potentially fouling marine bacterial strains is shown in Table II. Among different copper compounds commonly used as antifoulants (Yebra *et al.*, 2004), copper(I) oxide was chosen as the positive control in the antimicrobial assay due to its activity profile against the strains selected here. Other well known antifouling copper salts, such as CuSO_4 and CuCl_2 , were also tested in a wide range of concentrations, but the bacterial strains were remarkably resistant to these salts (data not shown).

Seven out of the 39 extracts of marine organisms had high activity against the tested bacterial strains, 10 extracts presented mild activity, 14 extracts showed low activity, and the other 8

extracts had negligible activity (Table II). Among the soft corals, the extracts of *Pseudopterogorgia elisabethae* (chemotype 1 and chemotype 2), *Eunicea knighti*, *Eunicea fusca*, *Eunicea succinea*, *Eunicea laciniata*, and *Eunicea* sp. 2 showed high to mild activity, respectively, and *Eunicea tayrona* and *Muriceopsis* sp. had low or no activity. The extracts of the sponges, *Topsentia ophiraphidites*, *Agelas citrina*, *Neopetrosia carbonaria*, and *Monanchora arbuscula* strongly inhibited bacterial growth, *Cliona tenuis*, *Ptilocaulis wallpersi*, *Iotrochota imminuta*, *Svenzea tubulosa*, *Oceania peltata*, and *Neopetrosia muta* were mildly inhibitory, while *Neopetrosia proxima*, *Neopetrosia rosariensis*, *Lissodendoryx colombiensis*, *Agelas conifera*, *Dragmacidon reticulata*, and *Biemna cribaria* weakly inhibited bacterial growth. The other six sponges had no remarkable activity. The extracts of algae showed low inhibition of bacterial growth. *Laurencia obtusa* was mildly active against 3 out of the 12 bacterial strains used. The extract of *Palythoa caribbaeorum* was mildly active against 4 out of the 12 bacterial strains used.

Extracts of *Pseudopterogorgia elisabethae* produced the largest inhibition zones and inhibited the widest range of strains; they have not been studied previously for their antibacterial or antifouling activity against bacteria isolated from a community associated to the surface of marine organisms. However, *Pseudopterogorgia elisabethae* has been widely investigated for the presence of diterpenes, mainly pseudopterogens, and related compounds that have proven activity as anti-inflammatory compounds (Correa *et al.*, 2009). In Colombia, two chemotypes with different and characteristic contents of pseudopterogens and secopseudopterogens have been reported as the result of our research and several new compounds have been isolated (Duque *et al.*, 2006). The antibacterial activity profiles of both chemotypes were very similar in the number of strains as well as in the strength of the activity, probably

Table II. Antifouling activity of extracts of marine invertebrates.

Sample	Growth inhibition of colonizer marine bacterial strains											
	1	2	3	4	5	6	7	8	9	10	11	12
Soft corals												
<i>Pseudopterogorgia elisabethae</i> (SA)	+++	-	-	++	N.E.	-	++++	-	+++	++++	-	++++
<i>Pseudopterogorgia elisabethae</i> (P)	+++	-	-	++++	N.E.	-	++++	-	++++	-	++++	++++
<i>Eunicea knighti</i>	+	-	++	+	++	-	++	++++	-	-	+	++++
<i>Eunicea succinea</i>	+	-	+	-	+	-	-	-	+++	+++	-	-
<i>Eunicea fusca</i>	++++	-	+	-	-	-	++++	++++	++	-	++	+++
<i>Eunicea</i> sp. 2	++	-	-	-	-	-	-	-	++	-	-	-
<i>Eunicea tayrona</i>	+	N.E.	+	-	+	+	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.
<i>Eunicea laciniata</i>	++	-	++	-	+	-	++	+++	+	++	+	++
<i>Erythropodium caribaeorum</i>	-	-	-	-	+	-	-	-	-	+++	-	++++
<i>Muriceopsis</i> sp.	-	-	-	-	-	-	-	-	-	-	-	-
Sponges												
<i>Biemna cribaria</i>	-	-	-	-	++	-	-	-	-	-	-	-
<i>Iotrochota imminuta</i>	+	-	++	++	++	-	-	-	+	++++	-	-
<i>Drumacidon reticulata</i>	-	-	-	-	+	-	-	-	++	-	-	-
<i>Oceanapia peltata</i>	-	-	-	-	+++	-	-	-	-	-	-	++++
<i>Cinachyrella kuekenthali</i>	-	-	-	-	+	-	-	-	-	-	-	-
<i>Spirastrella coccinea</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cliona tenuis</i>	-	-	-	-	-	-	+++	-	-	-	-	+++
<i>Cliona delitrix</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Agelas conifera</i>	+	-	-	++	-	-	-	-	++	-	-	-
<i>Agelas citrina</i>	++++	-	-	++++	++++	-	-	-	+	+	++	+++
<i>Monanchora arbuscula</i>	++++	-	+	++++	-	-	+	-	++	+	-	-
<i>Svenzea tubulosa</i>	++++	-	-	-	-	-	-	-	++++	-	-	-
<i>Neopetrosia proxima</i>	-	-	-	-	-	-	-	-	-	+++	-	-
<i>Neopetrosia subtriangularis</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Neopetrosia carbonaria</i>	++++	-	-	++++	++	-	-	-	++++	-	-	-
<i>Neopetrosia rosariensis</i>	-	-	-	-	-	++++	-	-	-	-	-	-
<i>Xestospongia muta</i>	-	-	-	-	-	-	-	-	+	+++	-	-
<i>Petrosia pallasarca</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Ptilocaulis wallpersi</i>	++++	-	-	+++	-	-	-	-	++	-	-	-
<i>Topsentia ophiraphidites</i>	++++	+	-	++++	++++	++++	++++	-	++++	+	++++	++++
<i>Lissodendoryx colombiensis</i>	-	-	-	-	-	++	+	-	-	++	++	-
<i>Amphimendon caycedoy</i>	-	-	-	-	-	-	-	-	-	-	-	-
Zoanthid												
<i>Palythoa caribbaeorum</i>	++	-	-	-	++	-	++	-	-	-	-	++
Algae												
<i>Laurencia catarinensis</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Laurencia microcladia</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Laurencia majuscula</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Laurencia flagelifera</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Laurencia obtusa</i>	+	-	++++	-	-	-	+	-	-	-	-	-
<i>Peyssonelia capensis</i>	-	-	-	-	-	+	+	-	-	-	-	-
Cu ₂ O	++	++++	+++	-	++	++	++++	++	+	++++	++	++++

The inhibitory activity is expressed as the diameter (in mm) of the inhibition zone 48 h after the test. -, no inhibition; +, 6.2–8.1; ++, 8.2–10.1; +++, 10.2–12.1; +++++, >12.1; N.E., not evaluated. Disc diameter, 5.2 mm. Concentration of extracts, 300 µg disc⁻¹. Positive control, 30 µg disc⁻¹ Cu₂O. Discs with the mixture of solvent extraction were used in each Petri dish as a control.

due to the presence of pseudopterosin-type compounds in both chemotypes.

The extracts of the *Eunicea* species, one of the most common genera of soft corals present in the Caribbean Sea, showed strong inhibition of bacterial growth. *Eunicea knighti*, *Eunicea fusca*, and *Eunicea laciniata* have the most interesting activity profiles according to the number of strains inhibited as well as the strength of the inhibitory effect. Studies on these soft corals identified them as a rich source of cembranoid and fuscoid di-terpenes, and some sesquiterpenes (Rodriguez, 1995). Several biological activities, such as antimicrobial, cytotoxic, and acetylcholine inhibitory activities, were reported for compounds isolated from soft corals belonging to this genus (Berrue and Kerr, 2009). Several cembranolides exhibiting cytotoxic and antibacterial activity have been isolated from *Eunicea succinea* (Rodriguez, 1995). *Eunicea fusca* contains fuscoides A–D, *i.e.* glycosidically bound diterpenes with skeletons related to lobane and eudesmane, which have an interesting anti-inflammatory activity (Jacobson and Jacobs, 1992).

The extract of *Topsentia ophiraphidites*, the most active one of all of our antifouling samples reported here, is a rich source of oxylipins, steroids including steroid sulfates, and nitrogen-containing steroids (Luo *et al.*, 2006; Alvi *et al.*, 1991). Although no studies have been done on the antibacterial activity of these compounds, they have proven antihelmintic and cytotoxic activities. Several 3- β - Δ^5 -steroids were identified by us in previous chemical studies in specimens collected at the Colombian Caribbean Cost (Echigo *et al.*, 2006).

The extract of the marine sponge *Agelas citrina* had some of the stronger inhibitory activities among the invertebrates tested here. Inhibition of four out of the 12 strains evaluated, as well as a mild inhibition of three other strains can be observed (Table II). On the other hand, *Agelas conifera* exhibited a low growth inhibitory effect on three strains. The Caribbean *Agelas* sponges are one of the most studied genera with a wide variety of metabolites of mixed metabolic origin (*i.e.* terpenoids, pyrrol and 2-aminoimidazole alkaloids, and halogenated compounds) and with a wide range of reported biological activities. Bromoageliferin and other 2-aminoimidazole derivatives inhibit biofilm formation by two planktonic bacterial strains of *Pseudomonas aeruginosa* (Hugens *et al.*, 2007). Agelasine D and its synthetic

analogues were shown to have antifouling activity against the barnacle *Balanus improvisus* (Sjögren *et al.*, 2008). Previous studies on *Agelas conifera*, a rich source of pyrrolidine alkaloids, revealed an inhibitory effect of these compounds on the settlement of *Vibrio harveyi* (Kelly *et al.*, 2003). As far as we know, *Agelas citrina* has not been subjected to chemical studies.

The antibacterial activity of four extracts of sponges of the genus *Neopetrosia* (previously classified as *Xestospongia*) and *Xestospongia muta* are presented in Table II. The strongest bacterial growth inhibitory effect was observed for *Neopetrosia carbonaria*, followed by *Neopetrosia rosariensis*, *Xestospongia muta*, *Neopetrosia proxima*, and *Neopetrosia subtringularis*, respectively. Several species of *Neopetrosia* collected in the Pacific Ocean, many of them unclassified, have been chemically explored in the search for bioactive compounds. From the studied species, original steroids (aragusterols), acetylenes, tetrahydrofurans, quinones and hydroquinones (xestoquinones), as well as a wide variety of alkaloidal compounds such as xestospongins, araguspongins, motuporamines, aaptamines, and renieramycins have been isolated (Laurent *et al.*, 2006; Cao *et al.*, 2005). *Neopetrosia carbonaria*, which also exhibited strong inhibitory activity among the sponges tested, contains the purple pyridoacridines and pyrroloacridines with strong cytotoxic activity (Thale *et al.*, 2002). *Neopetrosia proxima* and *Xestospongia muta* showed low inhibition of the marine bacteria tested here. From *Xestospongia muta*, the Caribbean barrel sponge, some acetylenic tetrahydrofuran compounds were isolated (Morinaka *et al.*, 2007). Extracts of *Neopetrosia proxima* collected in the Colombian Caribbean strongly inhibited human pathogenic strains of *Staphylococcus aureus*, *Streptococcus faecalis*, *Pseudomonas aeruginosa*, and *Escherichia coli* (Mora-Cristancho *et al.*, 2008). To our knowledge, no activity studies against marine colonizer bacteria or against other fouling organisms have been conducted with sponges of this genus.

The *Monanchora arbuscula* extract with strong antibacterial activity selective to strains 1 and 4 in the present study, contains mainly polycyclic guanidinic alkaloids with antibacterial, antifungal, antiviral and cytotoxic activities against tumour cell lines (Chang *et al.*, 2003). *Iotrochota imminuta* has not been the subject of chemical or antifouling studies, however, its potential as an antibacte-

rial agent has been evaluated (Mora-Cristancho *et al.*, 2008). Additionally, some bioactive aromatic alkaloids have been reported from other species of the same genera (Chan *et al.*, 1993).

Red algae of the genus *Laurencia* are cosmopolitan species with a wide distribution throughout the world. Species of this genus (order Ceramiales, family Rhodomelaceae) were proven to be a prolific source of structurally unusual and biologically active halogenated metabolites. We obtained similar results (Table II) for the extracts of *Laurencia catarinensis*, *Laurencia microcladia*, and *Laurencia majuscula*, and these are possible related to the common presence of elatol, characterized by chromatographic analyses of extracts of all these species (Lhullier *et al.*, 2010). The most potent antibacterial activity of the tested algal extracts was observed for *Laurencia obtusa*. The presence of elatol was reported for *Laurencia obtusa* collected in the Rio de Janeiro State of Brazil (Salgado *et al.*, 2008). For elatol, a potent antibacterial activity was previously reported against some marine bacteria and also against human pathogenic bacteria (Vairappan *et al.*, 2003). More complete investigations of the antifouling properties have been performed with *Laurencia rigida* extract, and the observed activity was attributed to the presence of elatol (König and Wright, 1997). On the other hand, in the tested *Laurencia flagelifera* extract, neither the presence of elatol or other related halogenated sesquiterpenes nor antibacterial activity were observed. A different activity profile was observed for the extract of *Peyssonelia capensis*; for this species there exists no previous report concerning antibacterial activity. Chemical investigation of *Peyssonelia* species collected in the Red Sea revealed the accumulation of two sesquiterpene hydroquinones, called peyssonol A and B, and these two compounds showed antiviral activity against HIV-1 and HIV-2 (Loya *et al.*, 1995).

The bacteria selected for the present study covered both, strains sensible and resistant to the extracts. The Gram-positive *Oceanobacillus iheyensis* (1), *Kocuria* sp. DQ448709.1 (4), and *Bacillus megaterium* (9) were sensitive, while the Gram-negative strains were either sensitive or resistant. The strain of *Alteromonas macleodii* (2) was strongly resistant against the tested extracts, and *Ochrobactrum pseudogrignonense* (6) was only susceptible to *Neopetrosia carbonaria* and *Topsentia ophiraphidites* extracts. On the other

hand, more sensible strains, such as *Vibrio campbellii* (7) and strain 12, were susceptible to a wide range of extracts which produced high to moderate growth inhibition zones. Some of the more interesting strains are 10 and 11, which were obtained from a gel immersed for 48 h, and thus possibly represent first colonizers on a surface. Strain 10 was inhibited by some of the active extracts of corals and sponges, while strain 11 was inhibited only by some of the octocoral extracts and by the *Topsentia ophiraphidites* and *Lyssodendoryx colombiensis* sponges extracts. No correlation between the source of a strain and inhibition can be derived from these data. However, it is clear that soft coral extracts are more inhibitory to the bacterial strains than sponges extracts.

In conclusion, fouling can be understood as a successive process that involves bacterial settlement and biofilm formation as the first steps. The settlement of macroorganisms is mediated by chemical cues originating mainly from the biofilm (Fusetani, 2004). Thus, inhibition of bacterial strains in colonized surfaces opens the perspective to find substances able to inhibit or delay the fouling process in its early stages. The results on the antibacterial activities presented here suggest the importance of carrying out studies on the chemical composition and antifouling activities of the soft corals *Pseudopterogorgia elisabethae*, *Eunicea knighti*, *Eunicea fusca*, *Eunicea laciniata*, and *Eunicea succinea*, and of the sponges *Topsentia ophiraphidites*, *Agelas citrina*, *Neopetrosia carbonaria*, *Monanchora arbuscula*, *Cliona tenuis*, *Iotrochota imminuta*, and *Ptilocaulis walpersii*. We have previously isolated several cembradiene diterpenoids (Tello *et al.*, 2009), a glycosylated pregnane, and dolabellanes from some of these species and a mixture of three known sterols from *E. succinea* (Cuadrado *et al.*, 2010) as compounds with antifouling activity against the bacteria tested here. Such studies are presently conducted in our group for *Eunicea fusca* and *Pseudopterogorgia elisabethae*.

Acknowledgement

This work was conducted as part of a bilateral (Brazil and Colombia) cooperative project financially supported by CNPq/MCT/Brazil (grant number 490151/2007-8) and COLCIENCIAS/Colombia (grant number 358-2007). Part of the work was also financed by COLCIENCIAS

(grant number 20101007015), by Fundación para la Promoción de la Investigación y la Tecnología del Banco de la República (grant number 200603 and 2057), and by DIB of the Universidad Nacional de Colombia (grant number 20101007467). The Ministerio de Ambiente, Vivienda y Desarrollo Territorial granted permission (permission No. 4 of 10/02/2010, addendum resolución 0306 of 22/02/2011) for collecting samples and per-

forming research on marine organisms collected at Santa Marta Bay and at the Rosario Islands, Colombian Caribbean. The Colombian authors greatly acknowledge Prof. Dr. Ute Hentschel from the University of Würzburg, Germany, Prof. Dr. Sven Zea from the Universidad Nacional de Colombia, and Dr. Diego L. Gil from INVEMAR (Santa Marta, Colombia) for their interest and assistance in this work.

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