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ORIGINAL ARTICLE

Diversity and biological activities of the bacterial community associated with the marine sponge *Phorbas tenacior* (Porifera, Demospongiae)

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Significance and Impact of the Study: This study presents the first report on the diversity of the cultivable bacteria associated with the marine sponge *Phorbas tenacior*, frequently found in the Mediterranean Sea. Evaluation of the antiplasmodial, antimicrobial and antioxidant activities of the isolates has been investigated and allowed to select bacterial strains, confirming the importance of *Proteobacteria* and *Actinobacteria* as sources of bioactive compounds.

Keywords
antimicrobial activity, antioxidant activity, antiplasmodial activity, associated bacteria, *Phorbas tenacior*, phylogenetic diversity, RAPD, sponge.

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Abstract

The diversity of the cultivable microbiota of the marine sponge *Phorbas tenacior* frequently found in the Mediterranean Sea was investigated, and its potential as a source of antimicrobial, antioxidant and antiplasmodial compounds was evaluated. The cultivable bacterial community was studied by isolation, cultivation and 16S rRNA gene sequencing. Twenty-three bacterial strains were isolated and identified in the *Proteobacteria* (α or γ classes) and *Actinobacteria* phyla. Furthermore, three different bacterial morphotypes localized extracellularly within the sponge tissues were revealed by microscopic observations. Bacterial strains were assigned to seven different genera, namely *Vibrio*, *Photobacterium*, *Shewanella*, *Pseudomonas*, *Ruegeria*, *Pseudovibrio* and *Citricoccus*. The strains affiliated to the same genus were differentiated according to their genetic dissimilarities using random amplified polymorphic DNA (RAPD) analyses. Eleven bacterial strains were selected for evaluation of their bioactivities. Three isolates *Pseudovibrio* P1Ma4, *Vibrio* P1MaNal1 and *Citricoccus* P1S7 revealed antimicrobial activity; *Citricoccus* P1S7 and *Vibrio* P1MaNal1 isolates also exhibited antiplasmodial activity, while two *Vibrio* isolates P1Ma8 and P1Ma5 displayed antioxidant activity. These data confirmed the importance of *Proteobacteria* and *Actinobacteria* associated with marine sponges as a reservoir of bioactive compounds.

Introduction

Marine sponges constitute a significant element in benthic communities in the world's oceans, in terms of both biomass and potential to participate in ecological processes. They are also recognized as a rich source of biologically active compounds (Blunt *et al.* 2012). Currently, five marine natural products or semisynthetic analogues isolated from sponges have been approved as therapeutics. Thirteen molecules are in clinical trials for various applications, mainly as anticancer agents, and one hundred are undergoing preclinical evaluation (Mayer *et al.* 2010). In the past decade, many studies have focused on the complex ecosystem sponge–microbial communities due to the association of sponges with dense and diverse microbial communities of the three domains of life (*Bacteria*, *Archaea* and *Eukarya*) (Taylor *et al.* 2007). The sponge-associated micro-organisms have revealed high densities,

amounting for some sponge species up to 37% of the sponge biomass (Vacelet 1975), which exceeded sea water concentrations by two to four orders of magnitude. More than 30 different phyla have so far been recognized in the domain *Bacteria* and *Archaea* as being associated with sponges (Webster & Taylor, 2012; Hentschel *et al.* 2012; Schmitt *et al.* 2012). However, <1% of these micro-organisms can be cultivated in laboratory conditions (Friedrich *et al.* 2001). Environmental stressors such as metal pollution, elevated sea water temperature or other climate changes and/or diseases were speculated as having a significant impact both on the symbiotic microbial community and on the health of sponges (Webster & Taylor, 2012). Some of isolated bacteria have been specifically associated with marine sponges and are absent from the immediate surrounding sea water, reinforcing the concept of sponge-specific microbes defined in the 1970s (Vacelet and Donadey 1977). Although the mechanisms of symbiotic associations and their benefits remain poorly understood, the role of bacteria in nutrient uptake (Wilkinson and Fay 1979), in the stabilization of the skeleton of the sponge (Wilkinson *et al.* 1981), but also in the chemical defence against predators or as antibiofouling agents has been suggested (Olsen *et al.* 2007). The suspected chemical defence role represented a real turning point in the search for new active marine biomolecules with potential medical and biotechnological applications. The major difficulty pertaining to the development of bioactive molecules concerns the possibility to ensure production at the industrial level. To solve this thorny problem of supply, different strategies have been explored, which have highlighted marine microbes as promising sources for drug leads. Several studies have demonstrated that numerous bioactive metabolites originally isolated from sponges were in fact synthesized or transformed by bacterial strains (Laroche *et al.* 2006; Quévrain *et al.* 2009). Therefore, the sponge-associated bacteria could constitute a renewable source of biomedical agents (Hentschel *et al.* 2001).

In our ongoing studies within the frame of the French program ECIMAR (Marine Chemical Ecology in the Mediterranean Sea), the Mediterranean demosponge *Phorbas tenacior* was selected after a screening of biological activities because its organic crude extract had revealed antioxidant and antiplasmodial activities. A great variety of original bioactive metabolites had been already reported from species of the genus *Phorbas* including alkaloids (Rudi *et al.* 1994), macrolides (Searle and Molinski 1995), terpenoids (McNally and Capon 2001; Rho *et al.* 2002; Jang *et al.* 2008; Zhang *et al.* 2008), steroids (Morinaka *et al.* 2007, 2009), peptides or modified peptides, as illustrated with anchinopeptolides isolated from *Anchinoe tenacior* (Casapullo *et al.* 1994), the former name of *P. tenacior* (Van-Soest 2007). However, although some of them revealed antifungal, cytostatic, antileukemic or repulsive compounds, neither antiplasmodial nor antioxidant compounds have been yet described from this genus. This report is the first on the cultivable microbial community associated with the *P. tenacior* sponge.

Thus, this study aimed at determining the phylogenetic affiliation of the bacterial community associated with the marine sponge *P. tenacior* by 16S rRNA gene sequencing of the cultivable strains. The localization of bacteria within the sponge tissues by ultrastructural microscopic observations was studied as well as the potential of the bacterial strains as sources of antioxidant, antimicrobial and antiplasmodial compounds.

Results and discussion

Electron microscopy observations

Using TEM and SEM observations, three bacterial morphotypes were detected within *Phorbas tenacior* and showed the presence of a low microbial density in the mesohyl of the sponge. All of them were extracellular and were localized in the sponge mesohyl (Fig. 1a). They had different shapes, sizes and intracellular characters.

Bacteria of morphotype B1 were rod-like, 1·9–2·5 μm in length and 0·8–1·1 μm in diameter (Fig. 1b,c). The cell wall had two distinct membranes and can be described as a Gram-negative type, matching with the high proportion of strains with this Gram staining isolated from this sponge (Table 1). The cytoplasm layer was narrow on the cell periphery. The nucleoid region was well developed, electron-dense and with an irregular network of filaments. B1 represents the most numerous bacterial morphotypes, and it was often localized near the granular cells.

The shape of morphotype B2 was ovoid, 0·6–0·7 μm in length and 0·3–0·37 μm in diameter (Fig. 1d,e). The cell wall displayed the same characteristics as that of morphotype B1. The zone of cytoplasm was transparent. The nucleoid region was narrow and filamentous.

Bacteria of morphotype B3 were very rare in the mesohyl. B3 had an unusual curved spiral form, characteristic of spirills (Fig. 1f). Their length was about 0·55 μm, and thickness, 0·25 μm. No flagella or pilus could be observed at the surface of the bacterial cell wall. Because of their scarcity, we only had SEM images of this morphotype, but could not provide any internal ultrastructure description. Spiral bacteria have been repeatedly observed as associated with marine invertebrates and also been previously observed in the mesohyl of several sponges (Vacelet and Donadey 1977; Ereskovsky *et al.* 2005, 2011; Taylor *et al.* 2007; Roue *et al.* 2010). They have been also described in other sponge species from the same genus

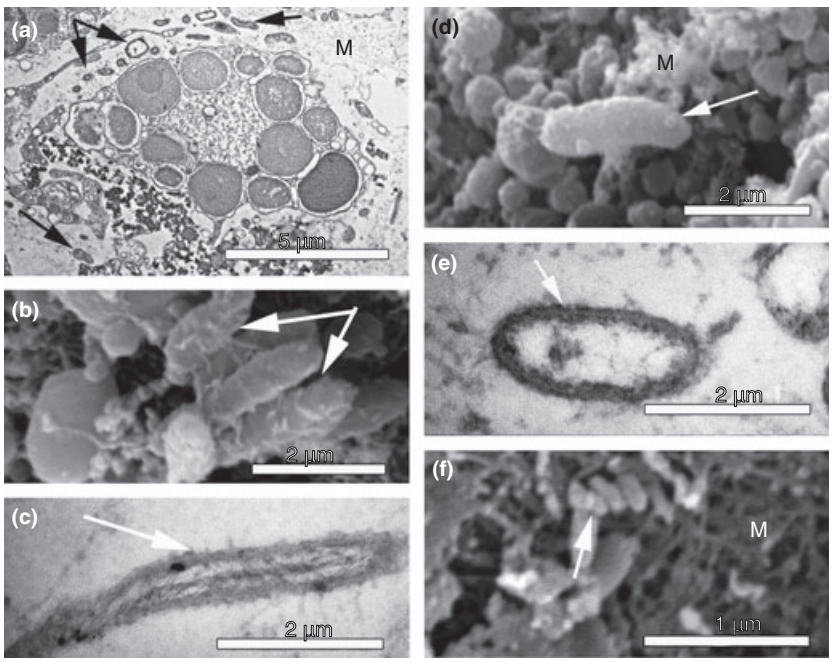

Figure 1 Scanning and Transmission Electron Microscopy (SEM and TEM) photographs of endobiotic bacteria (arrows) from *Phorbas tenacior*. (a) bacterial abundance and distribution in the mesohyl of *P. tenacior;* (b) SEM image of endobiotic bacteria of morphotype B1; (c) TEM image of endobiotic bacteria of morphotype B1; (d) SEM image of endobiotic bacteria of morphotype B2; (e) TEM image of endobiotic bacteria of morphotype B2; (f) SEM image of endobiotic bacteria of morphotype B3. C: sponge's cell, M: mesohyl.

Table 1 Characteristics of the bacteria isolated from *Phorbas tenacior* sponge sample

Medium	Strain	Description of colonies		Gram staining	16S rDNA fragment length (bp)	Sequences with best score in BLAST analysis (% Hom[e])	Class
		Colour[a]	Type[b]				
SWYE[c]	P1S1	Wh	R	–	701	*Vibrio* (99)	γ-Proteobacteria
	P1S2	Wh	R	–	1189	*Shewanella* (99)	γ-Proteobacteria
	P1S3	Wh	S	–	752	*Shewanella* (98)	γ-Proteobacteria
	P1S4	Ye	S	–	1446	*Shewanella* (98)	γ-Proteobacteria
	P1S5	Wh	R	–	658	*Vibrio* (100)	γ-Proteobacteria
	P1S6	Or	R	–	1543	*Vibrio* (99)	γ-Proteobacteria
	P1S7	Ye	S	+	1431	*Citricoccus* (99)	Actinobacteria
MB + nalidixic acid	P1MaNal1	Wh	M	–	1458	*Vibrio* (99)	γ-Proteobacteria
	P1MaNal2	Ye	R	–	1386	*Pseudovibrio* (99)	α-Proteobacteria
	P1MaNal3	Wh	R	–	1355	*Shewanella (*98)	γ-Proteobacteria
	P1MaNal4	Or	S	–	720	*Shewanella* (98)	γ-Proteobacteria
	P1MaNal5	Wh	S	–	1451	*Vibrio* (99)	γ-Proteobacteria
	P1MaNal6	Wh	S	–	656	*Vibrio* (100)	γ-Proteobacteria
	P1MaNal7	Br	R	–	1437	*Vibrio (*100)	γ-Proteobacteria
MB[d]	P1Ma1	Br	S	–	1232	*Ruegeria* (99)	α-Proteobacteria
	P1Ma2	Wh	R	–	760	*Pseudomonas* (99)	γ-Proteobacteria
	P1Ma3	Br	M	–	1381	*Pseudovibrio* (99)	α-Proteobacteria
	P1Ma4	Wh	R	–	1382	*Pseudovibrio* (99)	α-Proteobacteria
	P1Ma5	Ye	M	–	1225	*Vibrio* (99)	γ-Proteobacteria
	P1Ma6	Wh	R	–	1439	*Shewanella* (98)	γ-Proteobacteria
	P1Ma8	Wh	S	–	1455	*Vibrio* (99)	γ-Proteobacteria
	P1Ma9	Ye	R	–	746	*Photobacterium* (99)	γ-Proteobacteria
	P1Ma10	Or	S	–	1452	*Vibrio* (99)	γ-Proteobacteria

[a]Wh: white, Ye: yellow, Or: orange, Br: brownish.
[b]R: rough, S: smooth, M: mucoid.
[c]SWYE: sea water yeast extract.
[d]MB: marine broth.
[e]Hom: sequence homology.

Phorbas topsenti (Vacelet and Perez 2008). However, this bacterial morphotype was different from that observed in *P. tenacior* by its shape and size. This spiral morphotype, observed in the mesohyl of *P. tenacior* by electron microscopy, could not be observed for any of the isolates, suggesting its low abundance or its uncultivability in the laboratory culture conditions used along this study. One previous study suggested that several species spirochaetes might be present within the same sponge and sponge-specific (Neulinger *et al.* 2010). New approaches could be explored to enhance the bacterial cultivability as recently reported (Sipkema *et al.* 2011).

Isolation of bacteria

In parallel with these microscopic observations, serial dilutions of the sponge bacterial extract were performed and plated on sea water yeast extract (SWYE) and marine agar (MA) with or without nalidixic acid. Colonies were picked based on morphological characteristics and purified. Overall, using the three culture media, 23 strains were isolated from the *P. tenacior* sample (Table 1). The low cultured bacterial diversity may result from the isolation strategy. Various pigmentations of colonies were observed, and the majority of isolates stained Gram-negative except for the isolate P1S7, which stained Gram-positive. The obtained partial 16S rRNA gene sequences were analysed by comparison with the RDP and GenBank databases using the BLAST algorithm (Altschul *et al.* 1990). The phylogenetic tree inferred from 16S rRNA gene sequences for Gram-negative strains from this study together with those which presented the best BLAST score is shown in Fig. 2.

The length of the sequenced fragments and the results of the BLAST comparisons are presented in Table 1, with the highest BLAST score genus. The bacterial strains isolated from *P. tenacior* in this study could be assigned to two phyla *Proteobacteria* (Alpha-and Gammaproteobacteria classes) and *Actinobacteria*.

The high proportion of the *Proteobacteria* (96% of sponge isolates from this study) essentially included species of the genera *Vibrio* and *Shewanella*, both frequently encountered in marine environments and commonly found as epibionts at the surface of marine organisms (Yang *et al.* 2006; Hoffmann *et al.* 2012). Four of the 10 *Vibrio* isolates were obtained on a medium containing nalidixic acid, a compound known for its growth inhibition of some *Vibrio* species, confirming the natural resistance of *Vibrio* strains to nalidixic acid (Lajnef *et al.* 2012). Representatives of both genera showed to produce a wide variety of bioactive molecules with antifouling, antioxidative or antibacterial properties (Bhattarai *et al.* 2007; Nishida *et al.* 2007; Al-Zereini *et al.* 2010; Mansson *et al.* 2011). Three bacterial strains (P1Ma4, P1Ma3 and P1MaNal2) were affiliated to species of the genus *Pseudovibrio*, which has been commonly found in several marine invertebrates and may be involved in a symbiotic relationship with the sponge (O' Halloran *et al.* 2011; Margassery *et al.* 2012). Previous studies have demonstrated by fluorescence *in situ* hybridization a vertical transmission of *Pseudovibrio* bacteria between the larvae and the sponge, thus suggesting a symbiosis relationship (Enticknap *et al.* 2006; Schmitt *et al.* 2008).

On the basis of the 16S rRNA gene sequencing, the *Actinobacteria* strain P1S7 displayed 99·8% similarity to that of *Citricoccus* strains. Members of the *Citricoccus* genus have been reported from very diverse habitats including medieval wall painting (Altenburger *et al.* 2002), desert soil in Egypt (Li *et al.* 2005), bioreactor for saline wastewater (Meng *et al.* 2010) and shallow marine sediment (Kalinovskaya *et al.* 2011). However, this is the first report of a *Citricoccus* strain associated with a marine sponge.

Despite variability in morphology, several strains were assigned by molecular identification to the same genus. Indeed, 10 bacterial strains were identified as *Vibrio* species, six bacterial strains were assigned to the genus *Shewanella*, and three bacterial strains were assigned to the *Pseudovibrio* genus. Because 16S rRNA gene sequencing only provides information on the genus identification of sponge isolates, we performed a randomly amplified polymorphic DNA (RAPD) analysis (Fig. 3) to discriminate between the isolates belonging to the same genus (*Vibrio*, *Shewanella* and *Pseudovibrio*), which could be visualized by dendrogram analyses (Fig. 4b). While many of the *Vibrio* isolate RAPD profiles (Fig. 3a) shared common bands, the dendrogram analysis (Fig. 4a) demonstrated the presence of two clusters, divided into two subclusters: cluster I (Ia: strains P1Ma8, P1S1, P1S5, P1MaNal5 and P1MaNal6; Ib: strain P1Ma5) and cluster II (IIa: strain P1Ma10; IIb: strains P1MaNal7, P1S6 and P1MaNal1). Similarly, the RAPD analysis (Fig. 3b) discriminated two clusters among the six *Shewanella* isolates (Fig 4b): cluster I (strains P1Ma6, P1S2, P1S3 and P1S4) and cluster II (strains P1MaNal3 and P1MaNal4); and two clusters among the three *Pseudovibrio* isolates (Fig 4c): cluster I (strains P1Ma4 and P1Ma3) and cluster II (strain P1MaNal2). Based on their RAPD profiles and 16S rRNA gene sequences, 11 bacterial isolates (*Citricoccus* P1S7, *Vibrio* P1Ma8, P1Ma5, P1Ma10 and P1MaNal1, *Shewanella* P1Ma6 and P1MaNal3, *Pseudomonas* P1Ma2, *Pseudovibrio* P1Ma4 and P1MaNal2, and *Photobacterium* P1Ma9) were selected for bioactivity assays.

Bioactivities of bacterial isolates

The sponge crude extract exhibited a positive antioxidant activity in the qualitative DPPH assay (data not

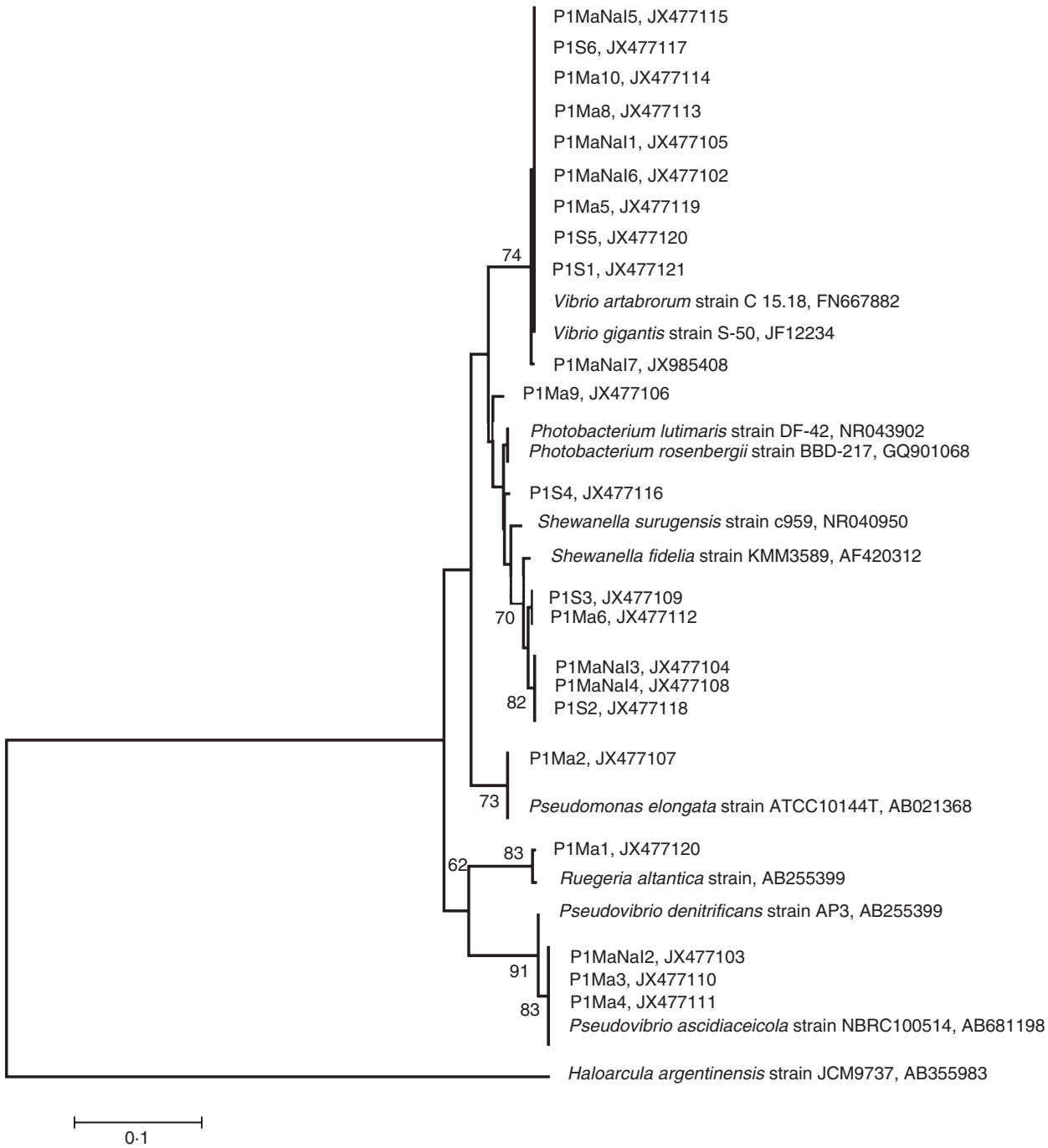

Figure 2 Phylogenetic analysis of 16S rRNA gene sequences from *P. tenacior* Gram negative bacterial isolates. The tree was constructed by using the neighbour-joining method (Bootstrap 1000). Genbank accession numbers are noted after the name of the strain. Bootstrap values over 70% are indicated. Scale bar: 0·1 substitutions per nucleotide position. The outgroup was the 16S rRNA gene sequence from *Haloarcula argentinensis.*

shown) and a significant antiplasmodial activity with 70% inhibition at 100 μg ml^{-1}. No antimicrobial activity (up to 100 μg ml^{-1}) could be detected in the *P. tenacior* extract against the environmental strains *Vibrio harveyi* (CIP 103192), *V. alginolyticus* (CIP 103336), *V. anguillarum* (CIP 63.36), *V. splendidus* (LGP32), *V. parahaemolyticus* (CIP 75.2), *Pseudoalteromonas distincta* (CIP 105340) and *P. atlantica* (CIP 104721) or against the pathogenic strain *Staphylococcus aureus* (CIP 4.83).

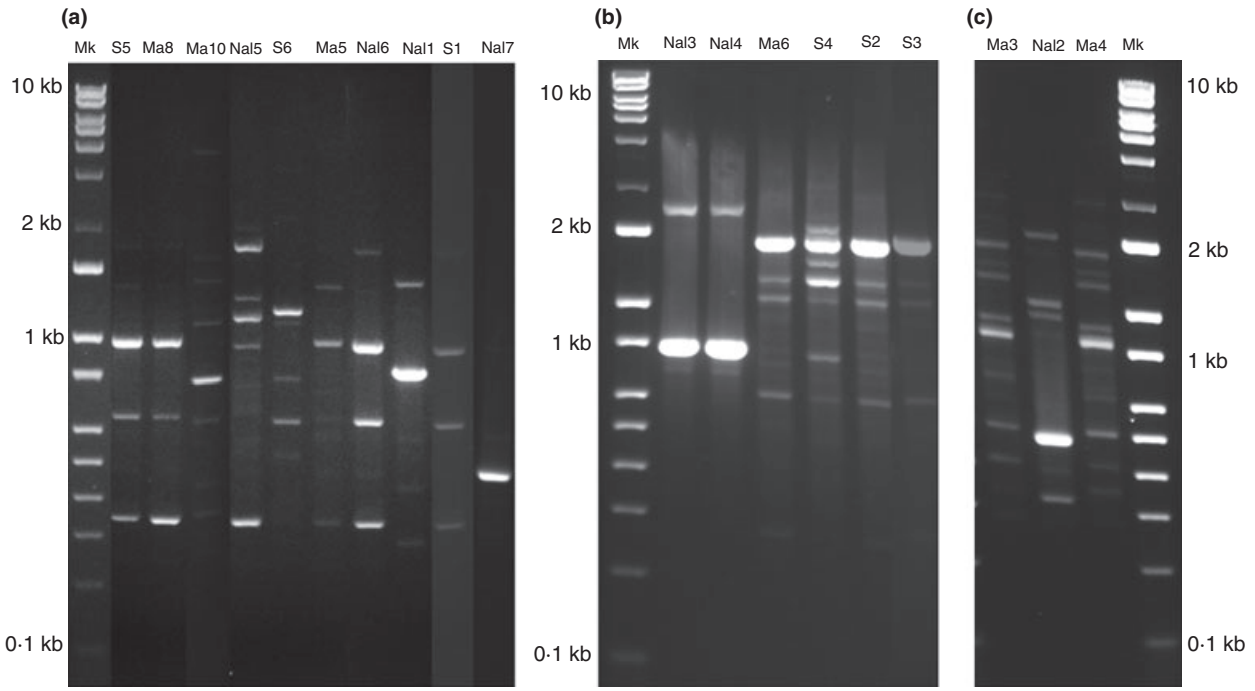

Figure 3 Diversity of *Phorbas tenacior* isolates RAPD profiles. Mk = ladder Log2 500 bp (Ozyme). (a) *Vibrio* isolates (S5 = P1S5; Ma8 = P1 Ma8; Ma10 = P1 Ma10; Nal5 = P1MaNal5; S6 = P1S6; Ma5 = P1 Ma5; Nal6 = P1MaNal6; Nal1 = P1MaNal1; S1 = P1S1; Nal7 = P1MaNal7); (b) *Shewanella* isolates (Nal3 = P1MaNal3; Nal4 = P1MaNal4; Ma6 = P1 Ma6; S4 = P1S4; S2 = P1S2; S3 = P1S3); (c) *Pseudovibrio* isolates (Ma3 = P1 Ma3; Nal2 = P1MaNal2; Ma4 = P1 Ma4).

Recently, micro-organisms associated with sponges have seen their interest renewed as a reservoir of new bioactive compounds. In particular, the antimicrobial compounds could be synthesized to ensure the defence against predators or bacterial competitions. Sponge and/or associated microbes have also the capacity to produce antioxidant compounds that can prevent protein or lipid sponge cell damages. Oxidative burst is a well-known mechanism of defence against infections by micro-organisms. It is well documented in plants (O'Brien *et al.* 2012), but it is not known whether this mechanism occurs in sponges. Nevertheless, the presence in *P. tenacior* of several isolates with antioxidant activity could suggest a way of protection of these bacteria against production of oxidative compounds by the host. In addition, the emergence of *Plasmodium falciparum* strains resistant to chloroquine has also stimulated the search of antiplasmodial molecules from sponge-associated bacteria.

Although the crude extract of *P. tenacior* did not show any antimicrobial activity, extracts of the bacterial strains cultivated in liquid medium were screened. *Citricoccus* sp. P1S7 strain revealed a moderate activity (diameter of inhibition between 3 and 6 mm) against several environmental microbes including *P. distincta* (CIP 10534), *V. splendidus* (LGP32), *V. algynoliticus* (CIP 103336), *V. anguillarum* (CIP 63.36) and *V. parahaemolyticus* (CIP 75.2). In addition, the two strains *Pseudovibrio* sp. P1Ma4 and *Vibrio* sp. P1MaNal1 exhibited a moderate antimicrobial activity (diameter of inhibition between 2 and 3 mm) against *V. parahaemolyticus* (CIP 75.2) and *P. atlantica* (CIP 104721). Thus, the bacterial community associated with *P. tenacior* may be involved in the host defence against predators and pathogenic organisms.

Because antiplasmodial and antioxidant activities were observed in the crude extract of *P. tenacior,* these activities were evaluated with the 11 different strains, selected based on their RAPD profiles.

Among the 11 dichloromethane/methanol bacterial extracts, two *Vibrio* isolates (P1Ma8 and P1Ma5) displayed antioxidant activity. Interestingly, they all belonged to the same RAPD cluster, suggesting that cluster I strains could have potential antioxidant capacities and could play a role in the sponge defence.

The antiplasmodial activity of the 11 bacterial strains was evaluated on the chloroquine-resistant *Plasmodium falciparum* strain FcB1. The crude extract of both bacterial strains *Citricoccus* P1S7 and *Vibrio* P1MaNal1 revealed 62 and 45% of inhibition of *P. falciparum* at 100 μg ml^{-1}, respectively. The CH_2Cl_2/MeOH extract of *Vibrio* P1MA8 and *Shewanella* P1MaNal3 revealed 20% of inhibition at 100 μg ml^{-1}, whereas no antiplasmodial activity could be detected in the other isolates. Our result is the first report

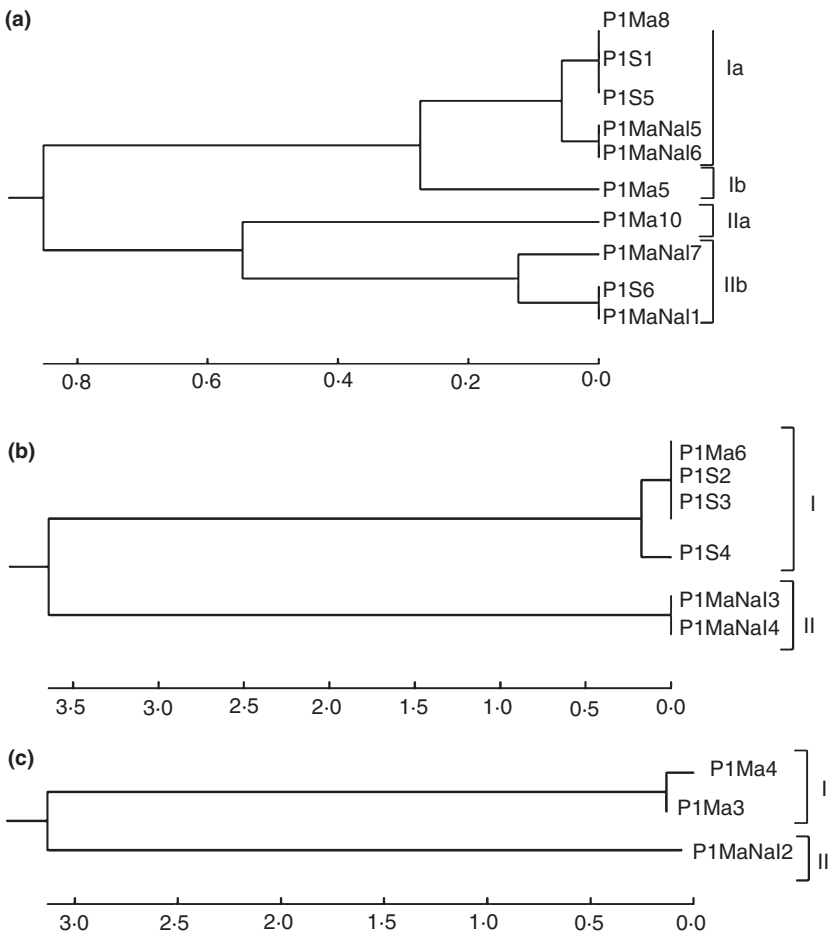

Figure 4 Clusters analysis using Fitch-Margoliash methods. (a) among the *Vibrio* isolates; (b) among the *Shewanella* isolates; (c) among the *Pseudovibrio* isolates.

of an antiplasmodial activity from a *Vibrio* strain. A further investigation revealed that the supernatant of *Citricoccus* P1S7 was responsible for the antiplasmodial activity with 100% inhibition against *P. falciparum* at 100 μg ml^{-1}, suggesting that the bioactive molecules were excreted. Thus, a bacterial origin for the antiplasmodial molecules was suggested. Antiplasmodial activity was also described in a recent study for the strain *Citricoccus alkalitolerans* isolated from marine sediments (Kalinovskaya *et al.* 2011).

In conclusion, two different isolates belonging to the genera *Vibrio* and *Citricoccus* (the *Proteobacteria* P1MaNal1 and the *Actinobacteria* P1S7, respectively) appeared as promising candidates for the discovery of antimicrobial and antiplasmodial compounds. In addition, one *Pseudovibrio* and two *Vibrio* isolates (the *Proteobacteria* P1Ma4, P1Ma8 and P1Ma5) could yield antimicrobial and antioxidant compounds, respectively. These data confirmed the interest of the *Proteobacteria* and *Actinobacteria* phyla as reservoirs of bioactive compounds.

Further chemical investigations are underway to identify the molecule(s) responsible for these activities and to clarify their role in the association with the sponge host.

Materials and methods

Sponge sampling

Phorbas tenacior (Topsent 1925) (formerly *Anchinoe tenacior*, Van-Soest 2007) (class Demospongiae, order Poecilosclerida, family Hymedesmiidae) is a thin blue encrusting sponge. This species is a sciaphilic Mediterranean sponge, found under rock overhangs and on falling in caves, down to 40 m depth.

Samples were collected in March 2010 by scuba diving in the north-western Mediterranean Sea (Marseille, France) at a depth of 15 m.

Transmission electron microscopy observations

After collection, small pieces of samples were immediately fixed in 2·5% glutaraldehyde in four volumes of cacodylate buffer (0·2 mol l^{-1}, pH 7·2–7·4) and five volumes of filtered sea water (1·120 mOsm, pH 7·2–7·4) for 1·5 h and then washed three times in cacodylate buffer for 10 min. Postfixation was performed in 2% OsO_4 solution in

filtered sea water for 2 h, followed by washing three times in cacodylate buffer for 15 min and dehydration in a graded ethanol series in distillated water. Prior to inclusion in the resin, the siliceous spicules were dissolved with 15–20% hydrofluoric acid in distilled water for 5 min. After desilicification and dehydration, the samples were embedded in Araldite© resin for ultrathin sections. Sections were cut with a Diatome 45° diamond (Ultracut microtome, Reichert, Depew, NY, USA). Ultrathin sections were counterstained with 2% uranyl acetate in a 50% ethanol solution and were observed under a Zeiss-1000 Transmission Electron Microscope (TEM) and LEO 910 at 75 kV with a Hitachi H7100 transmission electron microscope (TEM) equipped with a digital CCD Hamamatsu camera.

Scanning electron microscopy observations

After fixation, desilicification and dehydration, specimens were fractured in liquid nitrogen, critical-point-dried, sputter-coated with gold–palladium and observed under a Hitachi S 570 scanning electron microscope (SEM).

Isolation of cultivable bacteria

Sponges were thawed onto ice and cut aseptically into small pieces (4·5 g) using sterile scalpel. To reduce contamination, the sponge samples were washed in sterile artificial sea water (ASW). One piece of sponge was pressed through a 40-μm pore-size autoclaved plankton net with 10 ml of calcium and magnesium-free artificial sea water (CMF-ASW). Tissue extract was serially diluted (10^0–10^{-6}) in sterile CMF-ASW. One hundred microlitres of each dilution was plated in replicates on three solid media (Difco agar, 20 g l^{-1}): marine agar (MA, 2216, Difco), MA supplemented with nalidixic acid (0·001%) and sea water yeast extract (SWYE, 0·2% yeast extract, 2·34% NaCl, 0·15% KCl, 0·12% $MgSO_4$, 7 H_2O and 0·02% $CaCl_2$). Plates were incubated for 5–8 days at 15°C to reflect the natural conditions, and growth was monitored by observation of the colonies on the Petri dishes. Bacterial morphotypes were isolated and purified on MA. Bacterial strains were stored in a 40% glycerol solution in MB at −80°C.

PCR amplification of 16S rRNA genes

Colonies were picked using sterilized inoculating loop and dissolved in 30 μl of distilled water. Tubes were heated for 20 min at 110°C, followed by centrifugation at 4000 g for 2 min. The supernatant was placed in a clean tube. Aliquots (1 μl) of these samples were used as templates to amplify the 16S rRNA gene in a 50-μl PCR mixture with 0·5 10^{-6} mol l^{-1} universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Delong 1992), 2·5 10^{-3} mol l^{-1} of each deoxynucleoside triphosphate, 1·25 U *Taq* DNA polymerase and 1·5 10^{-3} mol l^{-1} $MgCl_2$ buffer (VWR). PCRs were conducted in a Veriti™ Thermal Cycler (Applied Biosystem, San Francisco, CA, USA) with initial denaturing step (95°C for 4 min) followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 55°C for 30 s and primer extension at 72°C for 30 s, and a final extension step was at 72°C for 7 min. Amplicons were separated by agarose (15 g l^{-1}) electrophoresis gel in TAE buffer containing 1× GelRed™ (Biotium, France) and visualized under UV transilluminator system.

Sequencing and phylogenetic analysis of 16S rRNA gene products

Amplicons were purified using the QIAquick purification kit (QIAGEN, Venlon, the Netherlands) and sequenced by Eurofins MWG Operon (Germany) or Beckman Coulter Genomics (Germany). The partial 16S rRNA gene sequences obtained were aligned using the BioEdit software (http://www.mbio.ncsu.edu) and Mega5 (http://www.megasoftware.net/) and compared using the BLAST algorithm with the sequences of GenBank (http://blast.ncbi.nlm.nih.gov) and RDP (http://rdp.cme.msu.edu/) databases (Altschul *et al.* 1990; Cole *et al.* 2003).

Randomly amplified polymorphic DNA analyses

The pure bacterial strains were grown in 2 ml of MB (Marine Broth 2216, Difco, France) and incubated at 15°C. After 24–48 h of incubation, cell cultures were centrifuged at 11 000 ***g*** during 20 min. Total genomic DNA was extracted from cell pellets with Purelink™ Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA). DNA was quantified using Nanovue Plus system (VWR).

PCR amplifications were performed in 50 μl of final volume reaction mixture containing 1·5 10^{-3} mol l^{-1} $MgCl_2$ buffer (VWR), 0·2 10^{-3} mol l^{-1} of each dNTP (Promega, Fitchburg, WI, USA), 2·5 units of Taq DNA polymerase (VWR), 0·6 10^{-6} mol l^{-1} of randomly amplified polymorphic DNA (RAPD) primers OPBC-18 (5′-GTGAAGGAGG-3′) and 2 μl of DNA extract (30 ng μl^{-1}). PCRs were conducted in a Veriti™ Thermal Cycler (Applied Biosystem) with initial denaturing step (95°C for 4 min) followed by 30 cycles of denaturation at 95°C for 1 min, primer annealing at 28°C for 30 s and primer extension at 72°C for 30 s, and a final extension step was at 72°C for 7 min. Amplicons were separated using agarose electrophoresis gel as described above. Gel analyses

were performed under image J software (http://rsbweb.nih.gov/ij/). Dendrograms were constructed using the Fitch–Margoliash method (PHYLIP software package, http://evolution.genetics.washington.edu/phylip).

Preparation of crude extracts of *P. tenacior* and bacterial strains

Freshly collected sponge samples (4·5 g) were extracted with a mixture of CH_2Cl_2/MeOH (1 : 1). After desalting the sample on SPE-C18 cartridge (Phenomenex, Torrance, CA, USA) by washing with distilled water, the crude extract was eluted with a CH_2Cl_2/MeOH (1 : 1) mixture and was evaporated under reduced pressure for the evaluation of its bioactivities.

Selected strains were cultivated in 20 ml of MB (Marine Broth) at 15°C under shaking (120 rpm) for evaluation of their bioactivities. After 48 h of growth, bacterial cultures were lyophilized and extracted with a mixture of CH_2Cl_2/MeOH (1 : 1) and evaporated. Crude extracts were stored at 4°C.

A culture of the bacterial strain *Citricoccus* P1S7 (2 l) in MB was centrifugated at 11 000 ***g*** at 4°C during 20 min. The supernatant was extracted with ethyl acetate, and the pellet, with CH_2Cl_2/MeOH (1 : 1) after being lyophilized.

Antiplasmodial assay

Plasmodium falciparum FcB1 strain was grown *in vitro* according to Trager and Jensen (Trager and Jensen 1976). Parasite culture medium contained RPMI 1640 medium (Life Technologies, Inc., Carlsbad, CA, USA), 25 10^{-3} mol l^{-1} Hepes, 27·5 10^{-3} mol l^{-1} $NaHCO_3$ and 11 10^{-3} mol l^{-1} glucose (pH 7·4) and was supplemented with 7·5% (v/v) compatible heat-inactivated human serum. Human red blood cells (RBC) were added at a haematocrit of 2%. The parasite culture was maintained at 37°C under an atmosphere of 3% CO_2, 6% O_2 and 91% N_2, with daily medium changes. The effect of the different extracts on the intraerythrocytic *P. falciparum* development was determined with cultures containing mixtures of all stages at a final parasitemia of *c.* 1% that were distributed in 96-well microplates, as previously described (Florent *et al.* 2001). The extracts in DMSO were added to the wells, in parasite culture medium, at final concentrations of 100 and 10 μg ml^{-1}. Parasites were then allowed to grow in a candle jar system, and after 24 h of culture, ^{3}H-hypoxanthine was added (0·5 μCi per well). After an additional 24-h incubation period, parasites were harvested on filters after a freeze-thawing cycle. Dried filters were submerged in a liquid scintillation mixture (OptiScintHisafe, Perkin Elmer, Waltham, MA, USA) and counted in a 1450 Microbeta counter (Wallac, Perkin Elmer, Waltham, MA, USA). Parasite growth inhibition was calculated from the parasite-associated radioactivity (incorporated into nucleic acids) in treated cultures compared with control cultures performed in the presence of equivalent amounts of DMSO, but without tested samples. Chloroquine was used as a positive control. Each extract was tested at least twice, in duplicates.

Antioxidant assay

The potential antioxidant activity of crude extracts was qualitatively evaluated using the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MI, USA) free radicals, visualized by spraying a purple DPPH solution (2 mg ml^{-1} in MeOH) on a thin-layer chromatography plate (Merck, Silica gel 60 F_{254}), where spot samples of a solution (10 mg ml^{-1}) were deposited. Immediate discoloration of DPPH around active samples revealed antioxidant activity. A solution of vitamin C (10 mg ml^{-1}) was used as a positive control.

Antimicrobial assay

Evaluation of the antimicrobial activity of the different bacterial strains was carried out using the agar double-layer diffusion method. Therefore, 5 ml of melted soft agar (6 g l^{-1} agar of MB with 6 g l^{-1} agar maintained at 42°C) was inoculated with 1–10 μl of exponential phase culture of the target strain (OD_{600} = 0·2–0·3) to a final OD_{600} = 0·0005 and poured into a Petri dish over MB (20 g l^{-1}). The target strains were *Vibrio harveyi* (CIP 103192), *V. alginolyticus* (CIP 103336), *V. anguillarum* (CIP 63.36), *V. splendidus* (LGP32), *V. parahaemolyticus* (CIP 75.2), *Pseudoalteromonas distincta* (CIP 105340), *P. atlantica* (CIP 104721) and *Staphylococcus aureus* (CIP 4.83). One bacterial colony of the potentially producing strains (*P. tenacior*) was deposited onto the plates. Incubation was carried out until observing a homogenous microbial lawn. Inhibition halos were measured in mm. The positive control was 2 μl of ampicillin (10 mg ml^{-1}).

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Conflict of Interest

The authors declare no conflict of interest.

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