RESEARCH ARTICLE

Exploring cultivable *Bacteria* from the prokaryotic community associated with the carnivorous sponge *Asbestopluma hypogea*

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Abstract

Combining culture-dependent and independent approaches, we investigated for the first time the cultivable fraction of the prokaryotic community associated with the carnivorous sponge *Asbestopluma hypogea*. The heterotrophic prokaryotes isolated from this tiny sponge were compared between specimens freshly collected from cave and maintained in aquarium. Overall, 67 isolates obtained in pure culture were phylogenetically affiliated to the bacterial phyla *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Firmicutes*. This cultivable diversity was lower than the prokaryotic diversity obtained by previous pyrosequencing study and comparable to that of another Mediterranean demosponge, the filter-feeding *Phorbas tenacior*. Furthermore, using fluorescence *in situ* hybridization, we visualized bacterial and archaeal cells, confirming the presence of both prokaryotes in *A. hypogea* tissue. Approximately 16% of the bacterial isolates tested positive for chitinolytic activity, suggesting potential microbial involvement in the digestion processes of crustacean prey by this carnivorous sponge. Additionally, 6% and 16% of bacterial isolates revealed antimicrobial and antioxidant activities, respectively. One *Streptomyces* sp. S1CA strain was identified as a promising candidate for the production of antimicrobial and antioxidant secondary metabolites as well as chitinolytic enzymes. Implications in the context of the sponge biology and prey-feeding strategy are discussed.

Introduction

Sponges are the simplest multicellular animals and are the most ancient metazoans, with a unique body plan built around a system of water canals. More than 8500 species have been so far described, based on both morphological and molecular characteristics (Van Soest *et al.*, 2012). They have colonized a wide variety of substrates (rocks, sediments, algae, bivalve shells…) from both deep seas and shallow waters. To date, 119 carnivorous sponges have been recorded in the deep-sea family of *Cladorhiza* (Van Soest *et al.*, 2012), of which 90% species belong to the genera *Asbestopluma*, *Cladorhiza*, and *Chondrocladia*. The capacity to capture small crustaceans for their nutrition is a unique feature among *Porifera* (Vacelet & Boury-Esnault, 1996), which are defined as sedentary filter-feeding metazoans (Bergquist, 1984). Therefore, these carnivorous sponge specimens might be the result of morphological and physiological adaptations to oligotrophic abyssal or cave environment (Vacelet & Duport, 2004).

One of these carnivorous species, *A. hypogea*, was discovered in 1996 for the first time in a shallow marine cave (at 17 m depth) of the Mediterranean Sea (La Ciotat, France), and several other specimens have been reported since, in the Mediterranean Sea, at depths from 100 to 600 m (Bakran-Petricioli *et al.*, 2007; Aguilar *et al.*, 2011). As *A. hypogea* could be maintained in aquaria, the capture and digestion processes could be studied for this tiny carnivorous sponge, which does not possess any digestive cavity. Electron microscopy observations revealed several microbial morphotypes, localized intracellularly within cell bacteriocytes and extracellularly in the sponge mesohyl, and representing 5–10% of the sponge volume, suggesting the involvement of associated micro-

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organisms in the host metabolism and digestion processes (Vacelet & Duport, 2004).

In a recent study of *A. hypogea*-associated prokaryotes carried out by our group, 454 pyrosequencing analysis of 16S rRNA gene sequences allowed the identification of two archaeal phyla and 20 bacterial phyla, among which the dominant ones were *Proteobacteria* and *Bacteroidetes*. A high number of different archaeal 16S rRNA gene sequences was detected, amounting to up to 40–50% of all sequence reads, including sequences affiliated to strains capable of ammonium oxidation. In addition, bacterial 16S rRNA gene sequences associated with sulfate-oxidizing/ sulfate-reducing *Bacteria* were reported, suggesting that the associated prokaryotes may be involved in nitrogen or sulfur cycling (Dupont *et al.*, 2013a). Specimens maintained in aquarium for 1 year shared sequences (OTUs) with the specimen from the cave. Specimens living in artificial laboratory conditions would therefore constitute a controlled model for future studies.

Complementary to former 454 pyrosequencing investigations of this carnivorous sponge prokaryote community, this study combines a culture-dependent approach, bioactivity assays, and microscopic observations to further assess and characterize the associated microorganisms from the carnivorous sponge *A. hypogea*. The diversity of the retrieved cultivable prokaryote community was also compared with that of the Mediterranean filter-feeding sponge *Phorbas tenacior* collected in the Marseille area (Dupont *et al.*, 2013b).

Although many methodological biases are attributed to cultivation-based approaches to study microbial communities, they nevertheless represent useful methods in microbial ecology because they reveal additional information to molecular-based approaches. Moreover, they provide access to the functional diversity and biotechnological potential of microbial strains. Due to the very low growth rate of many environmental microorganisms, less than 1% of the estimated microbial diversity is thought to be cultivable in laboratory conditions (Amann *et al.*, 1995). Among the 119 carnivorous sponge species currently described, no data are available concerning their associated cultivable microorganisms.

Using a culture-dependent approach, we investigated for the first time the diversity of prokaryotic strains associated with *A. hypogea*, obtained on different culture media. We compared the cultivable fraction of the microbial community associated with freshly collected or aquarium-maintained *A. hypogea* specimens. We also used fluorescence *in situ* hybridization (CARD-FISH) experiments to visualize the *Bacteria* and *Archaea* in the tissue of their sponge host. In addition, the antioxidant and antimicrobial activities of the isolates were evaluated for their potentialities as sources of bioactive compounds. The chitinolytic activity of isolates was also explored to obtain further insights into a potential microbial use of chitin derived from crustacean shell as carbon substrate.

Materials and methods

Sponge collection and seawater collection

Specimens of the Mediterranean carnivorous sponge *A. hypogea* (Class *Demospongiae*, order *Poecilosclerida*, family *Cladorhizidae*) were collected by scuba diving in the 3PP cave (17 m deep) off the coast of La Ciotat (France) in July 2009 (CQ) and May 2011 (CA, CB, and CC). Sponges were collected together with a fragment of their rocky wall cave substrate, to preserve the sponge integrity. Specimens were transported in 3 L of cave seawater to avoid contact with air. One liter of seawater (SW) was also collected in a sterile bottle in the 3PP cave surrounding the sponge specimens (May, 2011). The container was transferred immediately onto ice to the laboratory for isolation of associated microorganisms or tissue fixation for CARD-FISH analyses.

Isolation of cultivable *Bacteria*

Three specimens of *A. hypogea* collected in May 2011 were combined for isolation of their associated microorganisms (CA). In addition, three other sponges collected in July 2009 (CQ) were placed in aquarium for 18 months and monthly fed with *Artemia nauplii*. One month before their analyses, these aquarium-maintained sponges were starved.

The sponges were washed three times with sterile artificial seawater (ASW; 23.4% NaCl, 1.5% KCl, 1.2% $MgSO_4{\cdot}7H_2O$, 0.2% $CaCl_2$, $2H_2O$) before grinding the tissue with a sterilized mortar in a microtube. Three replicate *A. hypogea* samples, collected from either cave or aquaria, were homogenized in 500 μL of sterile artificial seawater, vortexed, and subjected to ultrasound for 30 s to disrupt most of the sponge cell membranes. After centrifugation for 30 s at 3000 ***g*** at 4 °C, serial dilutions (10^{-1} – 10^{-4} in artificial seawater) of the supernatant were used for the isolation of *Bacteria*.

Cave seawater (1 L) was sampled, and strains were isolated to be used as target environmental *Bacteria* in the antimicrobial assays. Filtration was performed through a 0.22-μm polycarbonate membrane (Millipore). Then, the membrane was cut aseptically and transferred into a sterile tube with addition of 5 mL of ASW and incubated overnight at 4 °C to detach microorganisms from filter. The resulting solution was then used without dilution or diluted to 10^{-2} in artificial seawater.

A volume of 100 μL of each dilution was plated in replicates on six solid media: marine agar (MA; Difco™; 40 g L^{-1}, pH 7.2), seawater yeast extract (SWYE; 1 L ASW, 0.2% Bacto™ yeast extract, pH 7.2), MA with 10 μg mL^{-1} nalidixic acid (MA-Nal), peptone–chitosan (PECH; 1 L ASW, 0.02% peptone from casein, 0.2% chitosan), delicious antibiotic (DA; 1 L ASW, 0.03% peptone from casein, 0.01% Bacto™ yeast extract, 0.001% D-glucose, 0.05% penicillin, 0.1% streptomycin (Sipkema *et al.*, 2011), or ammonium agar (Konneke *et al.*, 2005) (ACA; 1 L ASW, 0.007% ammonium chloride, 1 mL trace element solution and 1 mL of a mixture of vitamins (Widdel & Bak, 1992)). Unless otherwise specified, all reagents were from Difco (Fisher Scientific, France). The pH of media was adjusted to 7.2, and plates were prepared with 2% (w/v) agar. For ACA medium, plates were incubated under microaerobic conditions using bags (GenBag, Biomérieux, France). Plates were incubated at 15 °C in a dark chamber, and growth was monitored by weekly counts of the colonies on the plates for a period of 3 weeks. All the colonies formed on low-nutrient media (ACA, DA, PECH) and a selection of morphologically different colonies formed on high-nutrient media were subsequently re-isolated three times onto the same medium.

Antimicrobial assay

Evaluation of microbial antagonism was carried out using the agar double-layer diffusion method. Target strains were *Vibrio harveyi* CIP 103192, *V. alginolyticus* CIP 103336, *V. anguilarum* CIP 63.36, *V. mediterranei* CIP 103203, *V. parahaemolyticus* CIP 75.2, *Pseudoalteromonas distincta* CIP 105340, *P. atlantica* CIP 104721, *Staphylococcus aureus* CIP 4.83 (Institut Pasteur Paris), and *V. splendidus* LGP32 (Gift from Frédérique Leroux). Ten milliliters of melted soft agar (LB or MA with 6 g L^{-1} agar maintained at 42 °C) was inoculated with 1–50 μL of exponential-phase culture of the indicator strain (OD_{600} = 0.2 – 0.3) to a final OD_{600} = 0.0005 and poured into a Petri dish over 40 mL of solid LB or MA (with 20 g L^{-1} agar). Upon solidification of the soft agar, 10 μL from 24-h overnight cultures of each isolated bacterial strain was spotted onto the double-layer agar plate. Incubation was carried out until observing a homogenous microbial lawn. Inhibition halos were measured in mm (Supporting information, Fig. S1a). An antimicrobial activity was detected if the inhibition diameter against target strain was more than 10 mm, which corresponds to about half of the diameter obtained with positive controls (10 μL), ampicillin and streptomycin (10 μg mL^{-1}). Negative control was obtained by spotting 10 μL culture medium.

Antioxidant assay

Bacterial strains were grown in 50 mL of the isolation medium for 24–48 h (final OD_{600} = 1 – 2) at 15 °C with shaking. The whole culture was lyophilized overnight and extracted with 25 mL of a 1 : 1 mixture of dichloromethane/methanol. The potential antioxidant activity of the dichloromethane/methanol extracts was screened using the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma) free radicals. Crude extracts (100 mg mL^{-1}) were spotted on a thin-layer chromatography plate (Silica gel 60 F_{254}, Merck, France). After spraying of the purple DPPH solution (2 mg mL^{-1} in methanol), active samples were revealed by a color modification of the purple DPPH into yellow of the spot samples (Fig. S1b). A solution of vitamin C (10 mg mL^{-1}) was used as a positive control.

Chitinolytic assay

Screening of chitinolytic activity of bacterial isolates was performed using a solid medium containing colloidal chitin. This chitin solution was prepared from chitin of crab shell (Sigma-Aldrich) following the protocol described previously (Souza *et al.*, 2009). The minimal culture medium contained 1 L ASW (ASW; 23.4% NaCl, 1.5% KCl, 1.2% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCl_2$, $2H_2O$) and 1% colloidal chitin and was supplemented with 0.15% KH_2PO_4, 0.02% $Na_2HPO_4 \cdot 7H_2O$, 0.012% $MgSO_4$, 0.05% NH_4Cl, 0.0001% $FeSO_4 \cdot 7H_2O$, and 0.0001% $MnSO_4 \cdot 5H_2O$, with 2% of Bacto agar (Difco, France). The final pH was adjusted to 7.2. Bacterial strains were grown in their respective isolation medium and plated on chitin medium followed by incubation for 1 week at 15 °C. Extracellular chitinolytic activity was detected by the presence of clear zones around colonies (Fig. S1c). All experiments were duplicated, and one strain, *V. harveyi* CIP 103192, known for its chitinolytic activity, was used as positive control.

PCR amplification of 16S rRNA genes

Pure colonies on agar plates were picked with sterilized tooth pick and resuspended in 20 μL of sterile MilliQ water. This mix was heated for 20 min at 110 °C, followed by centrifugation at 4000 ***g*** for 2 min to remove cell debris. Supernatant was placed in a sterile tube for the following analyses. Aliquots (2 μL) of these samples were used to amplify the 16S rRNA gene in a 48-μL PCR mixture with 0.5 μM universal bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GG TTACCTTGTTACGACTT-3′) (Delong, 1992), 2.5 mM of each deoxynucleoside, 1.25 U *Taq* DNA

polymerase (VWR, France) and 1X PCR buffer with $MgCl_2$ (VWR, France). The PCR program was performed in a Veriti™ Thermal cycler (Applied Biosystem) as follows: intial denaturation at 95 °C for 4 min, 30 cycles of denaturation at 95 °C for 1 min, primers annealing at 55 °C for 1 min, elongation at 72 °C for 1 min and a final extension step at 72 °C for 7 min. For strain S1CA, direct colony PCR or PCR from genomic DNA failed to amplify the 16S rRNA gene sequences of this strain using the 1492R and 8F primers. Because the strain S1CA presented filamentous phenotype under the microscope, we suspected that it might belong to the *Actinobacteria* phylum. The cell wall of *Actinobacteria* is difficult to lyse in direct colony PCR, and the PCR efficiency is limited by their high G+C sequence content (Heuer *et al.*, 1997). Therefore, a PCR amplification was conducted using *Actinobacteria*-specific primers Com2xF (5′-AAACTCAAAGGAATTGACGG-3′) and Ac1186 (5′-CGCGGCCTATCAGCTTGTTG-3′) (Schäfer *et al.*, 2010). The PCR products were visualized and quantified on agarose (15 g L^{-1}) electrophoresis gel in TAE buffer containing 1X 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, Netherlands). Purified PCR products were cloned in the pGEM-T Easy vector (Promega, France) following the manufacturer's recommendations. Plasmids were purified using UltraClean™ Standard Mini Plasmid Prep Kit (Mo Bio Laboratories, inc.,) and quantified with a Nanovue Plus system (VWR). Sequencing was performed by Eurofins MWG Operon (Germany) or Beckman Coulter Genomics (Germany). Sequences were aligned using BioEdit software (http://www.mbio.ncsu.edu/bioedit/Bioedit.html) and compared using the blastn algorithm to sequences of GenBank (http://blast.ncbi.nlm.nih.gov), and phylogenetic identification was confirmed using rdp (http://rdp.cme.msu.edu) databases (data not shown) (Altschul *et al.*, 1990; Cole *et al.*, 2003). Phylogenetic trees were constructed using the neighbor-joining procedure by Mega5 software (http://megasoftware.net).

Nucleotide sequence accession number

All 16S rRNA gene sequences recovered from bacterial strains were deposited in the GenBank nucleotide sequences database under the accession numbers KF188468–KF188534 for *A. hypogea* sponge-isolated *Bacteria* and KF418793–KF418805 for *Bacteria* isolated from seawater.

In situ localization of microorganisms by CARD-FISH

For CARD-FISH experiments, two freshly collected specimens (CB and CC May 2011) were fixed in Bouin solution (5% glacial acetic acid, 75% saturated picric acid, 20% formalin; pH 4.0). After 24 h in fixative solution at 4 °C, sponge samples were washed three times with 70% ethanol and stored at 4 °C in 70% ethanol solution. Sponge tissues were dehydrated through a graded ethanol series (70–100%) and a final substitution, replacing ethanol with toluene 100% which is miscible with paraffin. Then, tissue fragments were embedded in paraffin (Paraplast plus, Leica, melting point: 56 °C). Histological sections (5 μM) were collected on Superfrost Plus slides (Thermo Scientific), deparaffinized with 100% toluene, and rehydrated through a decreasing series of ethanol (100 – 50%) and a final rinse with sterilized MilliQ water. Before hybridization, the tissue sections were pretreated with 10 mM HCl solution for 10 min to block endogenous peroxidases and rinsed with 20 mM Tris-HCl (pH 8.0) for 10 min. To allow access to ribosomal DNA for oligonucleotides probe, a treatment with proteinase K (0.5 μg μL^{-1}) was performed at 37 °C for 15 min, followed by incubation at 37 °C for 30 min with 10 mg mL^{-1} lysozyme. Hybridization was performed according to Quévrain *et al.* (2009) in a humidified preheated chamber with hybridization buffer (HB; 0.9 M NaCl, 0.02 M Tris-HCl, 0.01% SDS, 1% blocking reagent, 10% dextran sulfate, 35% formamide). To define optimal hybridization conditions, a gradient of 20–50% formamide was tested (Table 1). Since no archaeal strains were obtained in pure culture, we used a clone-FISH approach (Schramm *et al.*, 2002) to design a positive control for the archaeal probe (Table 1). The target archaeal sequence was selected based on the results of the former pyrosequencing analyses. In this previous study, an archaeal clone library was built by PCR amplification from gDNA extracted from *A. hypogea* using specific primers (Arch21F & Arch 958R) targeting the 16S rRNA gene of *Archaea* (Delong, 1992). This PCR product was cloned into pGEM-T easy vector (Promega), and the construction was transformed into *E. coli* DH5α competent cells (prepared from the MNHN collection of *Bacteria*). Archaeal sequence insertion was confirmed by PCR. Then, smears from two positive clones were prepared on Teflon-coated multiwell slides (Delta Microscopies, France), to serve as positive control for the archaeal probe. Bacterial smears of *E. coli* DH5α were used as a positive control for the universal eubacterial equimolar mix of EUB 338 (I, II, III) probe and as a negative control for the archaeal probe (Fig. S2). Archaeal smears of the strain *Haloarcula argentinensis* DSM12282 (DSMZ, Ger-

Table 1. Oligonucleotide probes used for *in situ* hybridization

Probes	Sequence (5′→3′)	Tm (°C)	Formamide (%)	Specificity	Position in *E. coli*
EUB I	GCTGCCTCCCGTAGGAGT[*,†]	62.2	30	*Eubacteria*	338–355
EUB II	GCAGCCACCCGTACGTGT[*,†]	62.2	30	*Eubacteria* (*Planctomycetales*)	338–355
EUB III	GCTGCCACCCGTAGGTGT [*,†]	62.2	30	*Eubacteria* (*Verrumicrobia*)	338–355
Non-EUB338	CTCCTACGGGAGGCAGC[*,†]	62.2	30	Nonsense *Eubacteria*	-
Cren554	TTAGGCCCAATAATCMTCCT[‡]	44.0	20	*Thaumarchaeota*	554–573

Tm, melting temperature.
*Amann *et al.* (1990).
[†]Daims *et al.* (1999)
[‡]Massana *et al.* (1997).

many) was used as a negative control for the bacterial EUB probe mix. Non-EUB338 nonsense probe was used as a negative control for background fluorescence due to unspecific binding. All HRP-conjugated probes were purchased from biomers.net (Germany). The thaumarchaeotal probe sequence was matched to sequences obtained from *A. hypogea* by 454 pyrosequencing (Dupont *et al.*, 2013a). Additionally, all probe sequences were evaluated for their coverage and specificity with the online tool probeCheck (Loy *et al.*, 2008) and are reported in Table 1.

Each tissue section or bacterial smear was covered with hybridization solution containing 50 ng of horseradish peroxidase-labeled probe (Table 1) in 150 mL of hybridization buffer and then hybridized as described previously for *Bacteria* (Quévrain *et al.*, 2009), but using a modified protocol without lysozyme for *Archaea* (Lloyd *et al.*, 2013). Amplification of the probe hybridization signal was detected with Alexa488 fluorophore (Invitrogen, France). Slides were coverslipped in the antifading mounting agent Fluoroshield containing DAPI (Sigma-Aldrich) and observed in wide-field epifluorescence on a Nikon ECLIPSE TE 300 microscope, with excitation and emission triple dichroic filter settings for DAPI and Alexa488 fluorophores.

Results

Isolation and identification of microbial strains from *A. hypogea*

To retrieve a diverse spectrum of prokaryotes, several media were used for strain isolation. Low-nutrient media (ammonium agar, delicious antibiotic and peptone–chitosan agar) yielded low numbers of colonies (<10 – 20 CFU/plate), whereas on high-nutrient media, (marine agar with or without nalidixic acid, SWYE) higher numbers of colonies were obtained (200 – 300 CFU/plate). Based on morphological differences, 55 and 12 isolates were isolated from the CA and CQ *A. hypogea* samples, respectively, after 2–3 weeks incubation on these solid media. Most of the 67 selected strains were isolated on the nutrient-rich MA and SWYE plates (55% and 25%, respectively, Table 2). Less than 3% of the isolates were obtained on low-nutrient media (Delicious Antibiotic, 2 of 67; ammonium agar, 1 of 67). Interestingly, 13% of the strains (9 of 67) were isolated on peptone–chitosan agar with low nitrogen content and chitin as a source of carbon. The 16S rRNA genes of the 67 isolates were partially sequenced and displayed 99–100% identity to sequences available in GenBank. Only bacterial strains and no archaeal strains were retrieved. The overall cultivable bacterial diversity which was obtained in our experimental conditions from the two specimens of carnivorous *A. hypogea* is shown in pie chart format (Fig. 1a and b) and in the phylogenetic tree (Fig. 2). These results should be considered qualitative and were also compared with previous results (Dupont *et al.*, 2013a) of pyrosequencing (Figs 1c–d and 3). However, short lengths of pyrosequencing reads make comparison with sequences from cultured *Bacteria* difficult below the class level.

For both *A. hypogea* samples, *Proteobacteria* (*Gamma-* and *Betaproteobacteria*) represented the large majority of isolates (with 62% and 67% for CA and CQ, respectively). The *Firmicutes* and *Bacteroidetes* accounted for 17–27% and 7–16% of the cultured isolates from *A. hypogea* CA and CQ specimens, respectively. In the CA sample (from natural cave habitat), approximately 7% of the isolates belonged to the *Actinobacteria* phylum. These isolates were classified (Fig. 2) into 17 distinct bacterial genera, with three most frequent genera: *Vibrio* (27%), *Bacillus* (21%), and *Pseudoalteromonas* (18%). While the phylum-level affiliations were maintained, there was significantly less consistency at lower taxonomic levels as illustrated at class level (Fig. 3). At genus level, only *Bacillus* was present in both cave and aquarium sponge samples (CA and CQ).

Additionally, the diversity of the cultivable bacterial community from the filter-feeding *P. tenacior*, obtained in a previous study (Dupont *et al.*, 2013b), is provided for comparison between demosponges with different lifestyles from the same geographic area (Fig. 3). For both sponges, the majority of isolates were affiliated to the *Proteobacteria*, most of them belonging to the *Gamma* class. Members of the *Alpha* class were isolated only from

Table 2. Taxonomic affiliation of the 67 *Bacteria* isolated from *Asbestopluma hypogea*

	Strain	Isolation medium	GenBank acc. no (length fragment in bp)	Sequences presenting the highest identity (%)
CQ sample from aquarium (July, 2009)	S1CQ	MA	KF188468 (1394)	*Bacillus aquimaris* JQ 030919 (99)
	S2CQ	SWYE	KF188469 (1325)	*Paracoccus carotinifaciens* NR 024658 (99)
	S3CQ	MA	KF188470 (981)	*Photobacterium aplysiae* NR 043188 (98)
	S4CQ	MA Nal	KF188471 (1533)	*Pseudomonas stutzeri* EU 520400 (99)
	S5CQ	MA	KF188472 (1427)	*Photobacterium frigidiphilum* NR 042964 (98)
	S6CQ	SWYE	KF188473 (1431)	*Staphylococcus equorum* JX 134628 (99)
	S7CQ	SWYE	KF188474 (991)	*Photobacterium aplysiae* NR 043188 (98)
	S8CQ	SWYE	KF188475 (1428)	*Photobacterium frigidiphilum* NR 042964 (98)
	S9CQ	MA	KF188476 (1375)	*Tenacibacculum gallaicum* NR 042631 (99)
	S10CQ	MA	KF188477 (1518)	*Tenacibacculum gallaicum* NR 042631(99)
	S11CQ	MA Nal	KF188478 (1341)	*Ruegeria scottomollicae* NR 042675 (99)
	S12CQ	MA	KF188479 (1334)	*Ruegeria scottomollicae* NR 042675 (99)
	S1CA	MA	KF188480 (616)	*Streptomyces microflavus* KC 788137 (98)
CA sample from cave (May, 2011)	S2CA	SWYE	KF188481 (1433)	*Vibrio gigantis* JF 412228 (99)
	S3CA	SWYE	KF188482 (1392)	*Salegentibacter mishustinae* AB 681203 (99)
	S4CA	MA	KF188483 (983)	*Pseudoalteromonas* sp. JQ 618825 (99)
	S5CA	MA	KF188484 (1416)	*Bacillus horikoshii* AB 617550 (99)
	S6CA	MA	KF188485 (1416)	*Bacillus horikoshii* AB 617550 (99)
	S7CA	DA	KF188486 (1410)	*Joostella marina* KC 534246 (96)
	S8CA	DA	KF188487 (1396)	*Tepidibacter* sp. AY 581271 (99)
	S9CA	SWYE	KF188488 (1407)	*Pseudoalteromonas citrea* NR 037073 (99)
	S10CA	SWYE	KF188489 (999)	*Pseudoalteromonas tetraodonis* KC 53435 (99)
	S11CA	MA	KF188490 (1378)	*Polaribacter dokdonensis* HE 584783 (99)
	S12CA	PECH	KF188491 (585)	*Shewanella pacifica* JQ 083320 (99)
	S13CA	MA	KF188492 (1435)	*Vibrio gigantis* JF 412228 (99)
	S14CA	MA	KF188493 (1431)	*Vibrio gigantis* JF 412228 (99)
	S15CA	SWYE	KF188494 (1362)	*Pseudoalteromonas* sp. JQ 618825 (98)
	S16CA	PECH	KF188495 (1411)	*Bacillus aquimaris* AB 617545 (99)
	S17CA	PECH	KF188496 (1414)	*Bacillus vietnamensis* HQ 699497 (100)
	S18CA	MA	KF188497 (1416)	*Bacillus horikoshii* AB 617550 (99)
	S19CA	MA	KF188498 (1001)	*Pseudoalteromonas issachenkonii* JX 867738 (99)
	S20CA	SWYE	KF188499 (1430)	*Enterovibrio calviensis* NR 041741 (97)
	S21CA	SWYE	KF188500 (1414)	*Bacillus vietnamensis* HQ 699497 (99)
	S22CA	PECH	KF188501 (1425)	*Vibrio gigantis* JF 412228 (99)
	S23CA	PECH	KF188502 (1415)	*Vibrio gigantis* JF 412228 (99)
	S24CA	MA	KF188503 (1410)	*Bacillus vietnamensis* HQ 699497 (100)
	S25CA	MA	KF188504 (562)	*Shewanella pacifica* JQ 083320 (99)
	S26CA	ACA	KF188505 (1425)	*Vibrio gigantis* JF 412228 (99)
	S27CA	MA	KF188506 (1344)	*Vibrio gigantis* JF 412228 (99)
	S28CA	SWYE	KF188507 (1398)	*Polaribacter dokdonensis* HE 584783 (99)
	S29CA	SWYE	KF188508 (1006)	*Pseudoalteromonas tetraodonis* KC 53435 (99)
	S30CA	MA	KF188509 (906)	*Pseudoalteromonas tetraodonis* KC 53435 (99)
	S31CA	PECH	KF188510 (1427)	*Vibrio celticus* FN 582227 (99)
	S32CA	MA	KF188511 (1400)	*Pseudoalteromonas atlantica* AB 049728 (99)
	S33CA	PECH	KF188512 (1415)	*Shewanella pacifica* JQ 083320 (99)
	S34CA	MA	KF188513 (1406)	*Pseudoalteromonas atlantica* AB 049728 (99)
	S35CA	MA	KF188514 (986)	*Pseudoalteromonas gracilis* AF 038846 (99)
	S36CA	PECH	KF188515 (1426)	*Vibrio celticus* FN 582227 (99)
	S37CA	MA	KF188516 (1417)	*Bacillus algicola* FR 775437 (99)
	S38CA	MA	KF188517 (1415)	*Bacillus horikoshii* AB 617550 (99)
	S39CA	MA	KF188518 (971)	*Pseudoalteromonas tetraodonis* KC 53435 (99)
	S40CA	SWYE	KF188519 (1413)	*Bacillus vietnamensis* HQ 699497 (99)
	S41CA	MA	KF188520 (1417)	*Vibrio tasmaniensis* GQ 455006 (99)

Table 2. Continued

Strain	Isolation medium	GenBank acc. no (length fragment in bp)	Sequences presenting the highest identity (%)
S42CA	MA	KF188521 (1398)	*Bacillus selenatarsenatis* EU 239470 (99)
S43CA	MA	KF188522 (1431)	*Vibrio celticus* FN 582227 (99)
S44CA	PECH	KF188523 (1374)	*Knoellia subterranea* EU 090713 (99)
S45CA	MA	KF188524 (1389)	*Vibrio lentus* AM 162659 (99)
S46CA	PECH	KF188525 (1372)	*Pseudoalteromonas atlantica* AB 049728 (99)
S47CA	PECH	KF188526 (1379)	*Massilia timonae* AY 445911 (100)
S48CA	MA	KF188527 (1386)	*Vibrio lentus* AM 162659 (99)
S49CA	MA	KF188528 (1416)	*Bacillus aquimaris* AB 617545 (99)
S50CA	MA	KF188529 (1394)	*Bacillus selenatarsenatis* EU 239470 (99)
S51CA	MA	KF188530 (876)	*Tepidibacter* sp. AY 581271 (99)
S52CA	MA	KF188531 (1431)	*Vibrio celticus* FN 582227 (99)
S53CA	SWYE	KF188532 (1433)	*Vibrio gigantis* JF 412228 (99)
S54CA	MA	KF188533 (1434)	*Vibrio lentus* AM 162659 (99)
S55CA	MA	KF188534 (1434)	*Vibrio gigantis* JF 412228 (99)

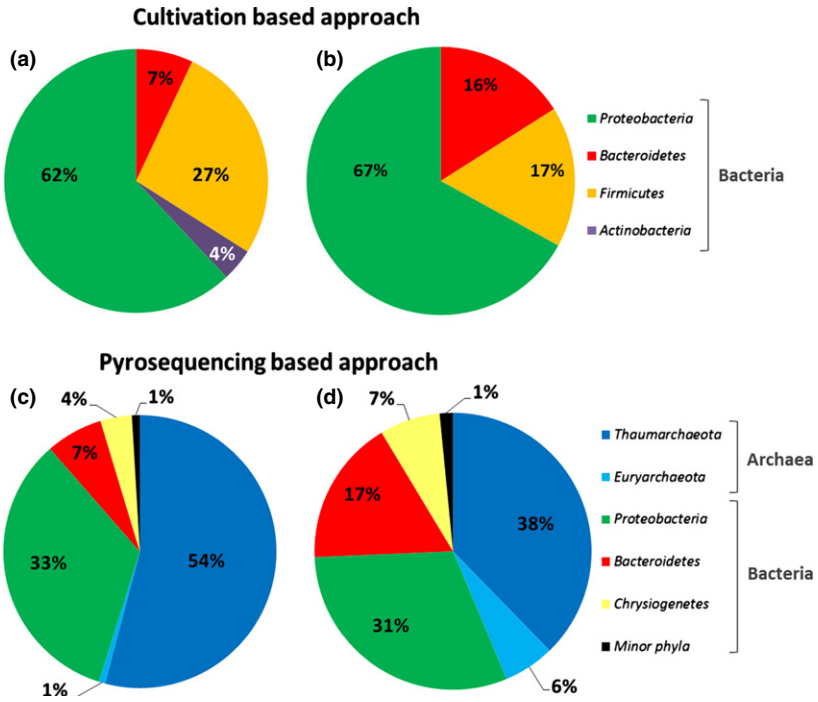

Fig. 1. Phylum-level composition of the prokaryote community associated with freshly collected or aquarium-maintained sponge, determined from culture-dependent or pyrosequencing approach. Data are expressed as relative abundance of the bacterial phyla cultured from (a) cave (three replicates CA, n = 55 sequences) and (b) aquarium (three replicates CQ, n = 12 sequences) sponge samples and compared to relative abundance of V6 hypervariable sequence reads from (c) freshly collected cave sponge (two replicates CA, n = 4055 sequences) and (d) aquarium-maintained specimen of *Asbestopluma hypogea* (two replicates CQ, n = 4723 sequences). (c and d from Dupont *et al.*, 2013a).

P. tenacior, while only one representative of the *Beta* class was obtained from *A. hypogea*. *Actinobacteria* were found in the same proportions from both carnivorous and filter-feeding sponges. Differences between experimental conditions and the relatively low sequence numbers obtained from each sponge species prevent detailed comparison at lower taxonomic level.

To obtain environmental *Bacteria* for competition assays with strains from *A. hypogea*, 13 environmental SW isolates were selected for sequencing of their 16S rRNA gene and comparison with sequences in GenBank using BLASTn algorithm. These isolates retrieved from cave seawater sample were affiliated to *Alpha-* and *Gammaproteobacteria* classes and distributed in seven genera, including *Pseudoalteromonas*, *Vibrio*, *Shewanella*, *Halomonas*, *Sphingomonas*, *Sulfitobacter*, and *Ruegeria*. Results of this phylogenetic affiliation are shown in Table S1.

Bioactivities of bacterial isolates

The 67 bacterial isolates obtained from *A. hypogea* were screened for their potential antioxidant, antimicrobial, and chitinolytic activities (Table 3).

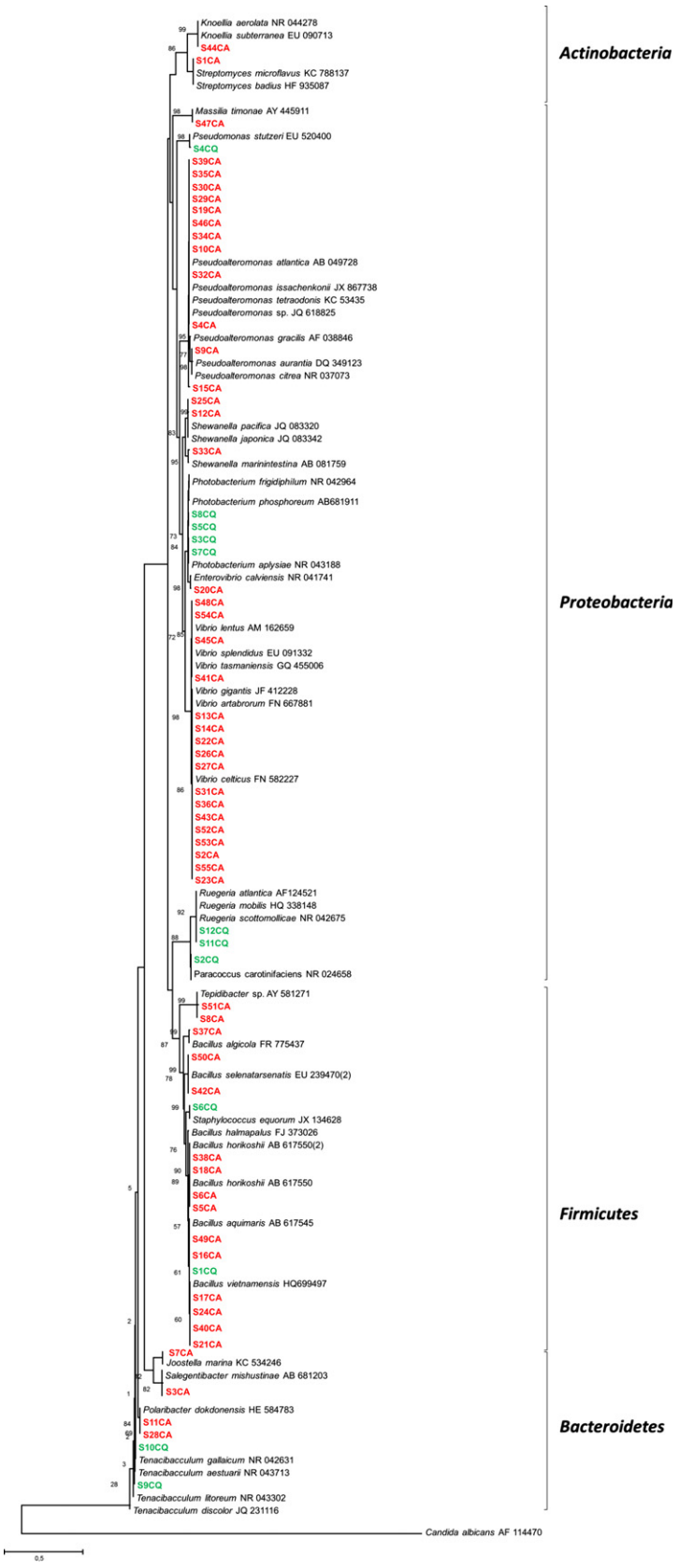

Fig. 2. Neighbor-joining phylogenetic tree from analysis of 16S rRNA sequences obtained from associated *Bacteria* cultured from a specimen *Asbestopluma hypogea* collected in July 2010 (in green) and in May 2011 (in red) and nearest neighbors obtain from GenBank (in black). The numbers at the nodes are percentages indicating the levels of bootstrap support, based on neighbor-joining analysis of 1000 resampled data sets. Only values > 75% are shown. The scale bar represents 0.2 substitutions per nucleotide position.

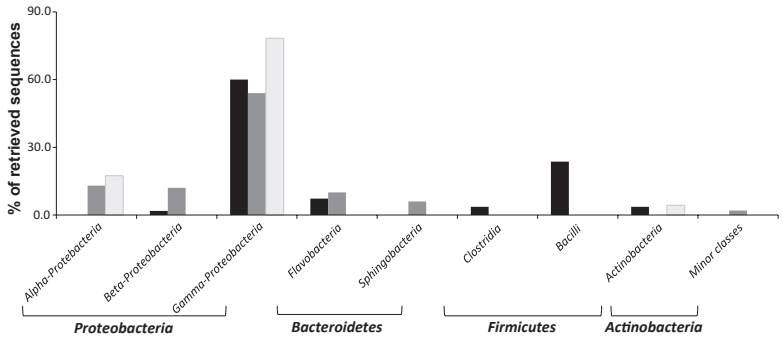

Fig. 3. Comparisons **of** the taxonomic affiliations at class level of *Bacteria* isolated from *Asbestopluma hypogea* cave sample (in black, CA), of sequences obtained by pyrosequencing (in gray, CA) (Dupont *et al.*, 2013a) and from Mediterranean sponge *Phorbas tenacior* (in white) (Dupont *et al.*, 2013b).

Overall, 11 strains displayed antioxidant activity (*Paracoccus* sp. S2CQ, *Pseudomonas* sp. S4CQ, *Photobacterium* sp. S7CQ, *Tenacibacculum* sp. S10CQ, *Ruegeria* sp. S11CQ, *Streptomyces* sp. S1CA, *Joostella* sp. S7CA, *Polaribacter* sp. S11CA, *Pseudoalteromonas* sp. S19CA, *Vibrio* sp. S31CA and *Bacillus* sp. S40CA), four of them (*Paracoccus* sp. S2CQ, *Streptomyces* sp. S1CA, *Joostella* sp. S7CA and *Vibrio* sp. S45CA) revealed an antibacterial activity against at least one target strain, and 11 isolates revealed a chitinolytic activity (*Paracoccus* sp. S2CQ, *Photobacterium* sp. S7CQ, *Photobacterium* sp. S8CQ, *Tenacibacculum* sp. S10CQ, *Streptomyces* sp. S1CA, *Joostella* sp. S7CA, *Polaribacter* sp. S11CA, *Pseudoalteromonas* sp. S19CA, *Vibrio* sp. S31CA, *Bacillus* sp. S40CA and *Vibrio* sp. S45CA). In addition, all isolates from sponge were tested against each other, but no activity was detected.

Three strains revealed significant activities in the three assays (*Paracoccus* sp. S2CQ, *Streptomyces* sp. S1CA, and *Joostella* sp. S7CA).

The strain *Streptomyces* sp. S1CA exhibited a strong activity against a wide range of bacterial strains including *Pseudoalteromonas distincta* CIP 105340, and *Vibrio parahaemolyticus* CIP 75.2. In addition, the *Streptomyces* sp. strain S1CA showed an antibacterial activity against *Vibrio* sp. S2SW, *Vibrio* sp. S3SW, *Ruegeria* sp. S13SW, and *Sulfitobacter* sp. S16SW strains isolated from seawater. Two other strains, *Paracoccus* sp. S2CQ and *Joostella* sp. S7CA showed an antibacterial activity against *Pseudoalteromonas distincta* CIP 105340.

CARD-FISH visualization of *Bacteria* and *Archaea* in sponge tissue

Using universal oligonucleotidic probes of the domain *Bacteria*, we localized the bacterial cells in *A. hypogea* tissue (Fig. 4a–c). Bacterial cells were widely distributed in all sponge tissue. They were observed enclosed in bacteriocytes or as isolated cells in the sponge mesohyl.

In addition, we visualized archaeal cells belonging to the *Thaumarcheota* phyla within *A. hypogea* tissue (Fig. 4d–f) by *in situ* hybridization with CREN554 probe. They were present in the sponge mesohyl, and were

Table 3. Antioxidant, antibacterial, and chitinolytic activities of *Asbestopluma hypogea* bacterial isolates

		Bioactivities		
Strain*	Identification	Antioxidant†	Antibacterial‡	Chitinolytic§
S2CQ	*Paracoccus* sp.	+	+¶	+
S4CQ	*Pseudomonas* sp.	+	–	–
S7CQ	*Photobacterium* sp.	+	+¶	+
S8CQ	*Photobacterium* sp.	–	–	+
S10CQ	*Tenacibacculum* sp.	+	–	+
S11CQ	*Ruegeria* sp.	+	–	–
S1CA	*Streptomyces* sp.	+	+¶,**,††	+
S7CA	*Joostella* sp.	+	+¶	+
S11CA	*Polaribacter* sp.	+	–	+
S19CA	*Pseudoalteromonas* sp.	+	–	+
S31CA	*Vibrio* sp.	+	–	+
S40CA	*Bacillus* sp.	+	–	+
S45CA	*Vibrio* sp.	–	+¶	+

(–): no active; (+) active

*From the screening of the 67 bacterial isolates, only those that exhibited a positive activity in at least one assay are presented in the table; †Using qualitative DPHH (2,2-diphenyl-1-picrylhydrazyl); ‡Using antagonism assays; §Using colloidal chitin; ¶Against *Pseudoalteromonas distincta* CIP 105340; **Against *Vibrio parahaeomolyticus* CIP75.2; ††Against environmental strains *Vibrio* sp. S2SW, *Vibrio* sp. S3SW, *Ruegeria* sp. S13SW, and *Sulfitobacter* sp. S16SW.

mostly distributed in the central area of the sponge body. The CREN554 probe hybridized specifically to clones containing the targeted thaumarcheotal 16S rDNA sequence fragments retrieved via pyrosequencing. Although no archaeal strains were recovered despite the use of a media containing ammonium, these results confirm the occurrence of *Thaumarchaeota* in the *A. hypogea* sponge as previously reported via pyrosequencing analysis (Dupont *et al.*, 2013a).

The prokaryotic cells were detected close to or inside sponge cells (inside bacteriocyte vacuoles in the case of most *Bacteria*). Only a small percentage (less than 5%) of the associated prokaryote phylotypes was affiliated to the *Thaumarchaeota* phylum, while the eubacterial cells were widely represented in all sponge tissues, representing 95% of the *in situ* hybridization signal.

Discussion

Sponges are known to host a wide range of microorganisms and have been considered as microbial fermenters (Hentschel *et al.*, 2006). To date, 30 bacterial phyla, two lineages of *Archaea*, and several types of microeukaryotic microorganisms have been identified associated with diverse filter-feeding sponges (Hentschel *et al.*, 2002, 2012; Taylor *et al.*, 2007; Hardoim *et al.*, 2009; Webster *et al.*, 2010; Lee *et al.*, 2011; Radax *et al.*, 2012; Schmitt *et al.*, 2012a, b; Webster & Taylor, 2012). Most of these phyla were identified by pyrosequencing of 16S rRNA gene fragments of the microbiome from filtering demosponges. Although these high-throughput sequencing techniques provide important information on microbial diversity associated with the sponge holobiont, the cultivation techniques reveal complementary results and represent one method to gain access to the biotechnological potential of the sponge-associated microorganisms. A recent pyrosequencing study by our group presented the first insights into the microbiome of the carnivorous sponge *A. hypogea* (Dupont *et al.*, 2013a). The present study constitutes the first report on cultivable *Bacteria* from a carnivorous sponge, obtained on low to high nutrient solid culture media.

In this study, cultivable microorganisms of two sponge samples of *A. hypogea* were isolated from the supernatant of homogenized sponge using both complex (marine agar with/or without nalidixic acid, seawater yeast extract) and specialized (peptone–chitosan, delicious agar and ammonium agar) media. Phylogenetic analyses of 16S rRNA gene sequences from the 67 isolates revealed that under these experimental conditions, the majority of cultivable *Bacteria* belonged to the *Proteobacteria*, *Firmicutes,* and *Bacteroidetes* phyla. Of the 67 isolates, only 13 were obtained from the low-nutrient media. Nevertheless, four strains belonging to four genera (*Joostella* sp. S7CA, *Tepidibacter* sp. S8CA, *Knoellia* sp. S44CA, and *Massilia* sp. S47CA) were obtained exclusively on these low-nutrient media.

All these isolates belonged to genera previously isolated from other demosponges (Taylor *et al.*, 2007; Sipkema *et al.*, 2011) including the filter-feeding *P. tenacior* collected in the same geographic area (Dupont *et al.*, 2013b). These results suggest that marine demosponges shelter similar cultivable microbial communities. From the aquarium-maintained *A. hypogea* specimen, we isolated only 12 strains. Because of the small number of isolates, this diversity cannot be compared to that of the freshly collected specimen. Only the genus *Bacillus* was identified from both sponge samples which may result from its capacity to form spores, possibly explaining that it is frequently retrieved from marine environments including many invertebrate hosts.

Results obtained via this culture-dependent approach are complementary to those obtained by 16S rRNA gene fragment pyrosequencing in *A. hypogea*, confirming the dominance in the microbiome of this sponge of the same bacterial phyla (*Proteobacteria*, *Firmicutes,* and *Bacteroidetes*). Increasing the number of culture conditions and media and systematically sequencing all isolates should increase the diversity of retrieved cultivable *Bacteria* and allow quantitative study of the cultivable community of this carnivorous sponge, as indicated by similar studies on the filtering sponge *Haliclona* sp. (Sipkema *et al.*, 2011).

The short length of pyrosequencing reads compared to the much longer 16S rRNA gene sequences obtained from cultured *Bacteria* make detailed comparison difficult. Although culture-dependent approaches have inherent biases due to lack of knowledge of growth requirements for environmental microorganisms, pyrosquencing approaches are not exempt of pitfalls due to PCR amplification and sequence quality selection processing biases (Amend *et al.*, 2010). Hence, there is a persistent need to use complementary approaches to explore the diversity of the complex microbiome of marine sponges. In this study, *Bacteria* were visualized within this carnivorous sponge tissue by *in situ* hybridization with universal probe mix for the domain *Bacteria*. Future experiments will involve hybridization with probes targeting specific bacterial taxons, to better understand their spatial distribution within the sponge.

No archaeal strains were isolated from *A. hypogea* although 454 pyrosequencing had previously revealed 16S rRNA gene sequences corresponding to ammonium-oxidizing *Archaea* (Dupont *et al.*, 2013a) and although a specific culture medium (ammonium agar) was used to target the microorganisms that use ammonium as energy source. Indeed, marine ammonium-oxidizing *Thaumarchaeota*

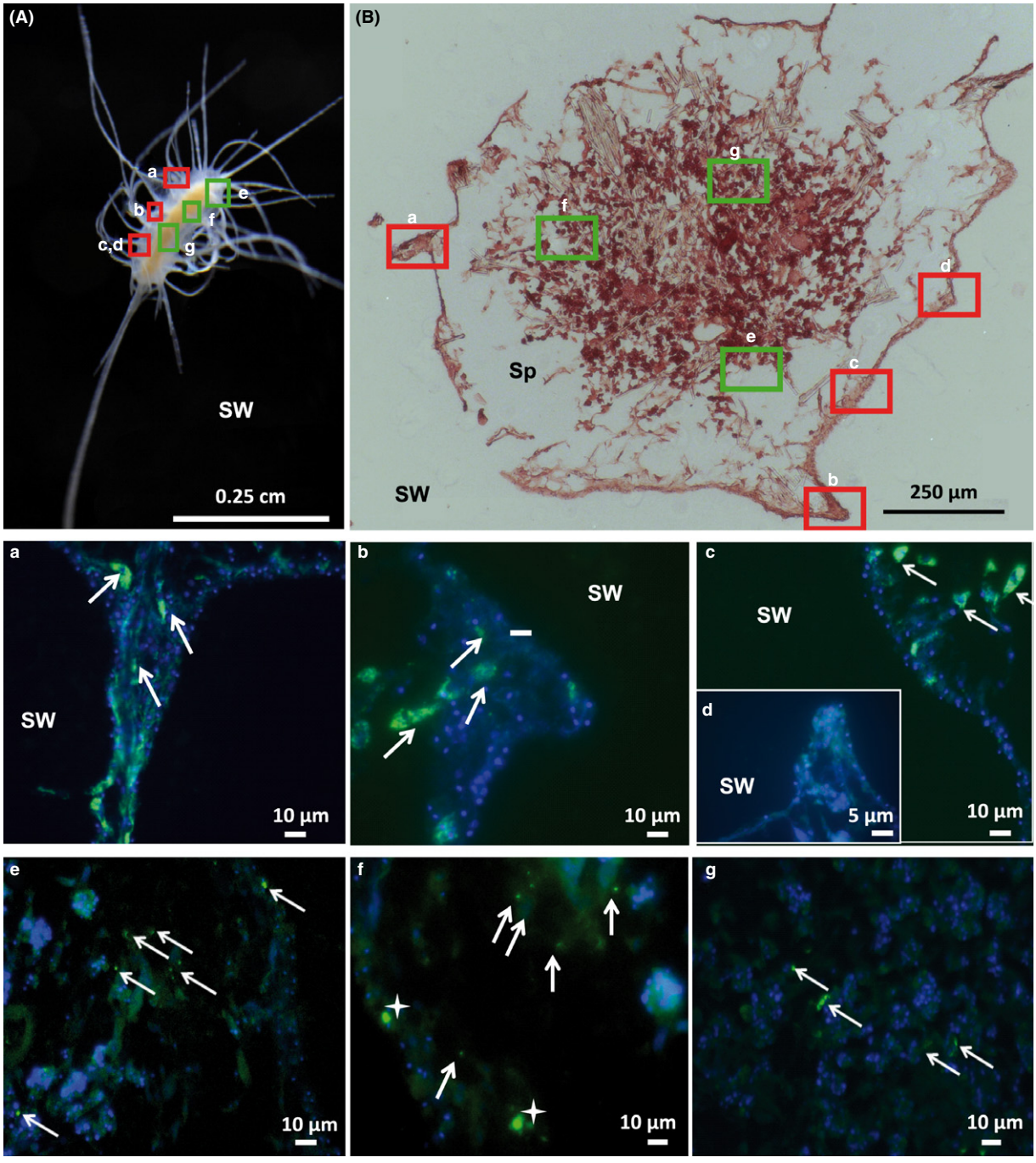

Fig. 4. Detection of sponge-associated *Bacteria* and *Archaea* within *Asbestopluma hypogea* tissues by catalyzed reported deposition fluorescence *in situ* hybridization (CARD-FISH) experiments. (A) Photography of a live sponge specimen. (B) Gram-stained histological cross-section of the sponge. Boxed insets indicate location of the areas analyzed in CARD-FISH. *Eubacteria* and *Archaea* belonging to the *Thaumarchaeota* phyla were visualized using CARD-FISH probes and localized relative to the DAPI-stained sponge cell nuclei. (a, b, and c) Tissue sections (at the base of the filaments) of *A. hypogea* hybridized with EUB 338 mix (EUB 338 I, II and III) (green) and stained with DAPI (blue). (d) Tissue sections of *A. hypogea* hybridized with the nonsense EUB probe. (e, f, and g) Tissue sections of *A. hypogea* (center of the body sponge) hybridized with Cren554 (green) and stained with DAPI (blue). Bar corresponding to 10 µm applies to all figure panels. Arrows indicate the bacterial or archaeal signals.

from aquarium gravel sediments have been previously cultivated in such medium (Konneke *et al.*, 2005). Several attempts have been made to improve the cultivability of *Archaea*, but are to date unsuccessful (Webster *et al.*, 2010; Hentschel *et al.*, 2012). Using CARD-FISH experiments, archaeal signals were observed within *A. hypogea,* confirming the presence of *Archaea* in *A. hypogea* tissues as inferred from pyrosequencing results (Dupont *et al.*, 2013a) although in lower abundance relative to the *Bacteria*. While the pyrosequencing data revealed the diversity of archaeal sequences (richness), the CARD-FISH experiment allowed estimation of the abundance of metabolically active archaeal cells (with high ribosomal content). It is possible that sponge-associated archaeal cells are very diverse with low abundance or in dormant state. Alternatively, for the pyrosequencing analysis, there might have been biases either in the PCR procedure (Klindworth *et al.*, 2013) or due to differences in 16S gene copy numbers among prokaryotic microorganisms (Kembel *et al.*, 2012). While *Bacteria* were preferentially localized at the periphery of the sponge, *Archaea* were mainly observed in central sponge tissue (where oxygen concentration may be lower). Recently, archaeal cells, in particular *Thaumarchaeota* Marine Group I cells, were also detected by *in situ* localization in the mesohyl of several filtering sponges, including the cold-water demosponges *Geodia barretti* and *Phakellia ventilabrum* (Radax *et al.*, 2012). Our inability to cultivate *Archaea* might be explained by lack of metabolic nutrients necessary for these associated microorganisms that could be provided by the sponge host, limited incubation time, or lack of viability of the archaeal cells outside their natural environment.

Cultivable *Bacteria* associated with sponges are of biotechnological interest as they are widely acknowledged as involved in host defense, especially through the production of antimicrobial metabolites (Taylor *et al.*, 2007; Piel, 2009; Thomas *et al.*, 2010). Approximately one-third of secondary metabolites isolated from cultured sponge-associated microorganisms are derived from the *Actinobacteria*, *Proteobacteria,* and *Firmicutes* bacterial phyla (Thomas *et al.*, 2010).

Overall, four strains belonging to the *Paracoccus*, *Streptomyces*, *Joostella,* and *Vibrio* genera revealed an inhibitory activity against the marine pathogen *V. parahaemolyticus* CIP 75.2 and the fish pathogen *P. distincta* CIP 105340 (Nelapati *et al.*, 2012). These results suggest that these associated *Bacteria* of *A. hypogea* could be involved in the defense mechanisms against marine pathogens. Antimicrobial activities of the sponge-derived strains, *Streptomyces* sp. S1CA and *Vibrio* sp. S45CA, were also observed against four environmental seawater-derived strains (*Vibrio* sp. S2SW; *Vibrio* sp. S3SW; *Ruegeria* sp. S13SW, and *Sulfitobacter* sp. S16SW). These results confirm the antimicrobial activities of marine environmental *Bacteria* affiliated to the genera *Streptomyces* and *Vibrio*, as has been widely documented (Selvin *et al.*, 2004; Pernice *et al.*, 2007; Dharmaraj & Sumantha, 2009; Wietz *et al.*, 2010).

As reported for terrestrial organisms (O'Brien *et al.*, 2012), sponges may produce reactive oxygen species (ROS) during the oxidative burst to defend against damages caused to the sponge by microbial pathogens (Regoli *et al.*, 2000). In our study, eleven bacterial isolates revealed an antioxidant activity demonstrated by DPPH assay. These isolates represent a potential microbial source of antioxidant molecules, which could be involved in the cross-talk between the sponge host and their associated *Bacteria*, to support survival of *Bacteria* within the sponge tissue.

Previous electron microscopy observations revealed an accumulation of microorganisms around the prey, suggesting their involvement in the digestive processes of *A. hypogea* (Vacelet & Duport, 2004). As this tiny carnivorous sponge captures small crustaceans, the sponge requires a mechanism to hydrolyze the chitinous walls of crustacean exoskeleton. A first step in digestion could be a breaking down of the prey by symbiotic microorganisms, previous to phagocytosis and intracellular digestion of prey fragments by sponge cells. Our data presented support this thought, although bacterial chitinases have not been evidenced.

Chitin is a naturally abundant linear polysaccharide polymer (β-1, 4-linked polymer of N-acetylglucosamine) which is present in the cell walls of fungi, algae, in the cuticle of arthropods, and the shells of mollusks. It is an important source of carbon and nitrogen for marine organisms (Gooday, 1990), especially for *Streptomyces* strains (Robbins *et al.*, 1988). In this study, all strains isolated from *A. hypogea* were evaluated for their capacity to hydrolyze the chitin. Eleven strains displayed a chitinolytic activity, including *Bacteria* belonging to the genera *Paracoccus, Tenacibacculum, Joostella, Photobacterium, Streptomyces,* and *Vibrio*. Strains of these two last genera are already known to produce chitinolytic enzymes (Robbins *et al.*, 1988; Aly *et al.*, 2011; Hoang *et al.*, 2011). Interestingly, strain S1CA, which displayed antimicrobial, antioxidant, and chitinolytic activities, belongs to the *Streptomyces* sp. genus, well-known to produce a large number of extracellular proteins and a large range of metabolites (Han *et al.*, 2009). Their potential presence should be confirmed in *A. hypogea* tissue to test hypotheses relative to their potential ecological role for this sponge.

The cultivable prokaryotic fraction of the carnivorous sponge *A. hypogea* was classified into the *Proteobacteria*, *Bacteroidetes, Actinobacteria,* and *Firmicutes* phyla. Bacterial and archaeal cells were present in the sponge tissue as visualized by CARD-FISH experiments but only *Bacteria*

could be cultivated. Of the 67 bacterial isolates obtained from two *A. hypogea* samples, 6% and 16% revealed antimicrobial and antioxidant activities, respectively, and 16% displayed chitinolytic activity. *Streptomyces* sp. S1CA was identified as a promising candidate for the production of secondary bioactive metabolites and the secretion of chitin degradation enzymes. Chemical investigations of active bacterial strains are underway to characterize the compounds responsible for these bioactivities.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Photographs of positive results in the antimicrobial (a), antioxidant (b), and chitinolytic (c) assays of *A. hypogea*.

Fig. S2. CARD-FISH using the Cren554 probe on a bacterial smear of the strain *E. coli* DH5α containing a pGEM-T plasmid with a thaumarchaeotal 16S rRNA gene sequence (a, positive control for the probe Cren554) and on a bacterial smear of the strain *E. coli* DH5α containing a pGEM-T plasmid with no archaeal 16S rRNA gene sequence (b, negative control for the probe Cren554). CARD-FISH using EUB 338 mix probes (EUB I + EUB II+EUB III) (c) and Non-EUB338 (d) on a bacterial smear of the strain *E. coli* DH5α. Red color was used for DAPI signals, green for the probe (Cren554, EUB 338 mix, or Non-EUB338) signals, and yellow for co-hydridization.

Table S1. Phylogenetic affiliation of 13 selected strains from seawater of the cave.