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Elodie Quévrain
Laboratoire Molécules de Communication et Adaptation des Micro-organismes, Muséum National d'Histoire Naturelle, Paris, France

Mélanie Roué
Laboratoire Molécules de Communication et Adaptation des Micro-organismes, Muséum National d'Histoire Naturelle, Paris, France

Isabelle Domart-Coulon
Laboratoire Molécules de Communication et Adaptation des Micro-organismes,Muséum National d'Histoire Naturelle, Paris, France Laboratoire de Biologie des Organismes et des Ecosystèmes Aquatiques, Muséum National d'Histoire Naturelle, Paris, France (former affiliation)

Marie-Lise Bourguet-Kondracki
Laboratoire Molécules de Communication et Adaptation des Micro-organismes, Muséum National d'Histoire Naturelle, Paris, France., bourguet@mnhn.fr

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ASSESSING THE POTENTIAL BACTERIAL ORIGIN OF THE CHEMICAL DIVERSITY IN CALCAREOUS SPONGES

Elodie Quévrain[1], Mélanie Roué[1], Isabelle Domart-Coulon[1, 2], and Marie-Lise Bourguet-Kondracki[1]

Key words: calcareous sponges, cultivable bacteria, antimicrobial activity, bacterial antagonisms, chemical mediation of interactions.

ABSTRACT

The chemodiversity and cultivable bacterial diversity of temperate calcareous sponges were investigated in a time series of collection of two sponges, *Leuconia johnstoni* (Baerida, Calcaronea) collected from the northeastern Atlantic Ocean and *Clathrina clathrus* (Clathrinida, Calcinea) collected from the northwestern Mediterranean Sea, using combined chemical and microbiological approaches.

Bacteria were visualized in tissue sections of these sponges with Gram staining and *in situ* hybridization. The sponge crude extracts revealed annually persistent biological activities against reference human pathogen strains: *L. johnstoni* extracts displayed antimicrobial activity against a Gram positive *Staphylococcus aureus* strain; *C. clathrus* extracts displayed a broad spectrum of antimicrobial activities against Gram positive *S. aureus* and Gram negative *Escherichia coli* strains as well as against the yeast *Candida albicans*. In order to determine the sponge or bacterial origin of the secondary metabolites isolated from the sponge holobiont, sponge cells and bacteria were separated *via* differential centrifugation of dissociated sponge suspensions and each fraction was screened for biological activity. The aerobic cultivable bacteria associated with these calcareous sponges were isolated, amounting to about $10^5 - 5 \times 10^6$ bacteria per sponge wet weight. Based on 16S rRNA sequence comparison, the bacterial community was found in both species to be composed mostly of Gammaproteobacteria (60-80%) and Alpha-proteobacteria (15%), with a few Firmicutes, Actinobacteria and Bacteroidetes. A range of biologically active compounds were purified from crude extracts of sponge or from culture broths of bacterial isolates. Ecological implications for the host sponge are being discussed based on localization and abundance of the producing bacteria *in situ* in the sponge tissue and on the co-detection of the chemical fingerprint of the bacterial metabolites in the host extracts. Some bacterial compounds were shown to have a role as chemical mediators of interactions within the sponge-associated bacterial compartment.

I. INTRODUCTION

The oceans which cover more than 70% of the earth's surface and contain more than 500,000 described species of plants and animals are a rich source of biodiversity, with representatives of every phylum, including 14 exclusively marine phyla out of the 35 animal phyla in the tree of life. This exceptional marine biodiversity has afforded an incredible chemodiversity, providing so far around 25,000 molecules with no equivalent in terrestrial natural products [5].

Sponges are sessile benthic organisms, with few physical defenses (in the form of mineralized spicules), which respond to ecological threats like predation, fouling or competition by producing a wide range of chemical defense molecules. These latter molecules, often structurally unique, have revealed pharmacological activities with valorization potential in the anticancer, antibiotic, anti-inflammatory, immuno-modulatory, and analgesic fields [31]. However, the development of these molecules into commercially available drugs is hampered by the limited sponge biomass. New biotechnological approaches are needed for solving the supply problem associated with the development of most marine drug candidates.

In the class Demospongiae, the associated bacteria hosted by these active filter feeders, constitute a persistent and stable community, with a sponge-specific assemblage different from seawater, which can amount up to 10^8-10^9 bacteria per gram sponge wet weight, corresponding to concentrations 100 to 1000 fold higher than in the bacterioplankton [23]. The

Paper submitted 06/25/13; accepted 07/18/13. Author for correspondence: Marie-Lise Bourguet-Kondracki (e-mail: bourguet@mnhn.fr).
[1] Laboratoire Molécules de Communication et Adaptation des Micro-organismes, Muséum National d'Histoire Naturelle, Paris, France.
[2] Laboratoire de Biologie des Organismes et des Ecosystèmes Aquatiques, Muséum National d'Histoire Naturelle, Paris, France (former affiliation).

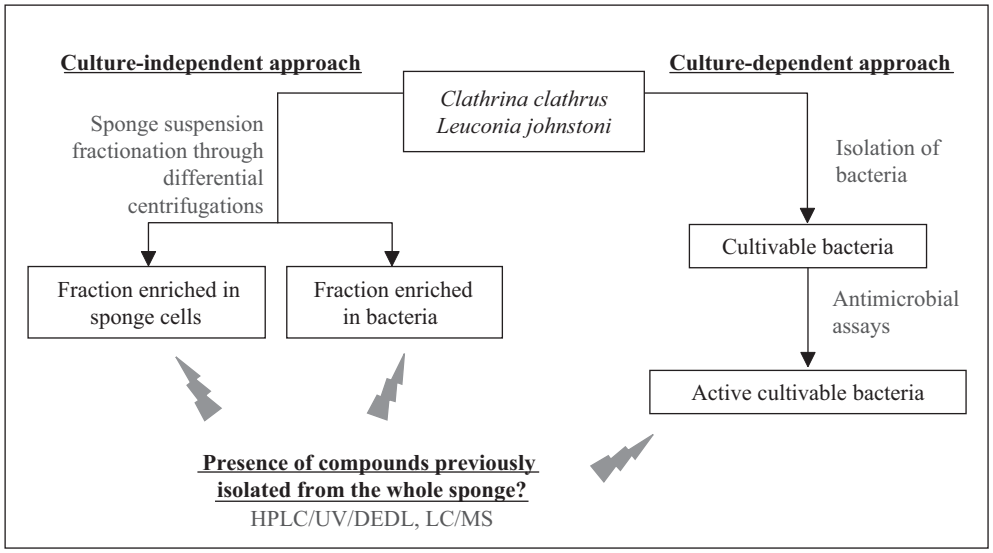


Fig. 1. Culture-independent and culture-dependent approaches developed to investigate the contribution of associated bacteria to the secondary metabolism of sponges.

structural similarity of many natural products isolated from demosponges to those isolated from bacteria has been considered as an indication of their putative bacterial origin [38]. Indeed, bacteria have long been proposed as responsible for the production of numerous natural products isolated from marine invertebrates. However, studies of invertebrate-bacteria associations for biotechnological purposes are faced with serious technical challenges, such as the general resistance of bacteria to isolation and propagation in microbiological culture, and the complexity of the marine microbial assemblages.

Compared to Demospongiae, calcareous sponges (Calcispongiae) have been barely studied as models, both in terms of their chemistry and their microbiology [44], despite the unique range of increasing complexity of their aquiferous system organization (from ascon to sycon, to leucon), which might affect the density and complexity of their associated bacterial community. Early electron microscopy studies have revealed the presence of diverse bacterial morphotypes in calcareous sponges. Their relatively low microbial density and diversity might represent an advantage to better understand the origin of the secondary metabolites they produce, and the putative role of their endosymbionts.

Here we provide an overview on culture-dependent and culture-independent strategies (Fig. 1) employed by our group to contribute to a better understanding of the origin of the secondary metabolites of temperate littoral marine calcareous sponges. We focused on the northeast Atlantic leuconoid sponge *Leuconia johnstoni* (Carter, 1871), collected off the coast of Concarneau (Brittany, France) – previously named *L. nivea* Grant 1826 *sensu* Bowerbank 1866 - and on the Mediterranean asconoid sponge *Clathrina clathrus* (Schmidt, 1864) collected from the coast of Marseille (France).

To solve the question of the origin of molecules produced by a sponge, the isolation of them from a pure culture of an associated microorganism still remains the most conclusive technique. However, it is well known that only a small fraction of marine microorganisms (0.01 to 1%) is currently cultivable under laboratory conditions [14, 49, 55]. Thus, cellular localization of compounds has been developed these last years to provide a first indication of their potential site of production, e.g. either host cells [15, 41, 42, 45, 46, 53] or associated microorganisms [4, 13, 26, 33, 51, 52]. It should be noted that this technique only provides circumstantial evidence, since the hypothesis of complete transport of the metabolites from one cell type to another cannot be excluded [47].

In this work, microscopy observations, including Gram staining and *in situ* fluorescence hybridization (CARD-FISH) of tissue sections with bacterial probes, were used to visualize bacteria in their calcareous sponge host. The antimicrobial activity of sponge crude extracts was tested against the Gram positive bacterial strain *Staphylococcus aureus* ATCC 6538, the Gram negative bacterial strains *Escherichia coli* ATCC 8739 and the yeast *Candida albicans* ATCC 66029. Differential centrifugation experiments were conducted to localize the biological activities in the sponge or bacterial fractions of dissociated sponge suspensions. Bacteria with the same spectrum of biological activities were isolated. One of the most active strains from *L. johnstoni*, *Microbulbifer* sp. L4n2, was chemically studied and the bacteria and its metabolites were detected *in situ* in the sponge host. The relationships between a frequent *Pseudoalteromonas* sp. bacterial isolate and the *Clathrina clathrus* sponge were investigated. Chemical study of cultures of a representative *Pseudoalteromonas* sp. strain led to the isolation of two bacterial metabolites which were also detected in the *C. clathrus* sponge by liquid chromatography – mass spectrometry (LC/MS) and were abundant

enough to be purified by HPLC chromatography. Implications for a food *versus* symbiotic nature of the interactions between these cultivable bacteria and their associated sponge are discussed.

II. MATERIALS AND METHODS

1. Biological Material

Specimens of *Leuconia johnstoni* were collected at the lower limit of the intertidal zone (accessible only for tide coefficients higher than 100) in a time-series of 13 collections from 2005-2009 off the coast of Concarneau (South Brittany, France), covering all seasons. Specimens of *Clathrina clathrus* were collected by scuba-diving at 5-20 m depth, off the coast of Marseille (Golfe du Lion, France) in a time series of 7 collections from 2007-2010, covering all seasons. Collected sponge fragments were transported to the laboratory in a closed container with ambient seawater from the site of collection. Fragments of different individuals were pooled, examined under a binocular to manually discard epibionts, and rinsed three times with 0.2 μm filtered seawater to remove most loosely attached surface bacteria. These samples were processed immediately, either for freezing prior to chemical analysis, or for bacterial isolation on microbiological media, or for tissue preservation in Bouin fixative for microscopy. Voucher specimens were deposited in the Porifera collection of the Muséum National d'Histoire Naturelle (France), under registration number *Leuconia johnstoni* C2009-1 to C2009-18 and *Clathrina clathrus* C2010-1.

2. Microscopic Observations to Visualize Bacteria in Sponge Tissue

Freshly collected sponge specimens were fixed and simultaneously decalcified for histology study in Bouin fixative (containing picric acid 0.9%, formol 9%, acetic acid 5%). Specimens were then washed extensively with 70% ethanol and stored at 4°C. Tissues were dehydrated in an increasing series of ethanol, substituted in xylene and embedded in paraffin. Histological sections (6-8 μm thick) were cut with disposable stainless steel blade on Leica RM2265 microtome and collected on Superfrost slides (DeltaMicroscopies), deparaffinized with xylene and rehydrated.

Gram staining of tissue sections was performed according to Martoja [30] to characterize the morphotype of bacteria within sponge host. This technique was also used for individual bacterial isolates smeared on a slide.

Catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) of tissue sections was performed according to protocol published in Quévrain *et al.* 2009 [40]. The probe EUB338 detects most bacteria except Planctomycetales and Verrucomicrobiales [11]. The NonEUB probe was used as negative control [1]. CARD-FISH experiments was also carried out with specifically designed oligonucleotidic probes, targeting the phylotypes of bacteria frequently isolated or exhibiting interesting biological activity. Simultaneous DAPI staining of DNA allowed to visualize in the tissue the spatial localization of bacteria relative to the sponge cell nuclei. All probes (HRP conjugated) were purchased from biomers.net (Germany). The hybridized DNA was observed with an Olympus epifluorescence microscope using filter sets for DAPI (BP365/12 FT396 LP397) and Alexa-Fluor 488 (BP450-490 FT510 LP515).

3. Screening of Biological Activity of Chemical Extracts or Isolated Compounds

Freshly collected *C. clathrus* sponges (total wet weight 40 g) were lyophilized (total dry weight 4.2 g), stored frozen and then extracted for 3 successive times, with a mixture of CH_2Cl_2/MeOH 1:1 (20 mL extraction followed by 15 min sonication, at room temperature). This protocol, standardized for all participants to the French ANR programm ECIMAR, allowed recovery of both apolar and polar compounds.

Freshly collected *L. johnstoni* sponges (about 20 g wet weight per sampling time) were stored frozen and then extracted overnight for 2 successive times, with CH_2Cl_2 at room temperature. This protocol for apolar compounds was selected after preliminary extractions with MeOH had failed to reveal biological activities.

Antimicrobial activities of extracts or isolated compounds were tested in the agar disk diffusion assay, in plates seeded with a lawn of reference pathogen bacteria *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 or the yeast *Candida albicans* ATCC 66029. Bactericide and bacteriostatic activities were recorded after 24 h incubation at 37°C by measuring the diameter of the inhibition zone around the disk imbibed with test compound (1 mg). Results were repeated in at least three independent experiments.

4. Fractionation of Dissociated Sponge Suspensions, to Separate Sponge Cells from Extracellular Sponge-Associated Bacteria

Rinsed sponge fragments (about 1 g total wet weight) were mechanically dissociated by pressing through 40 μm pore-size autoclaved plankton net, in 0.2 μm filtered calcium and magnesium free artificial seawater (CMF-ASW), after the method modified from Wilson 1907 [56] to prevent reaggregation of cells. The dissociated sponge suspensions were filtered through a 40 μm mesh sterile nylon sieve to remove large spicule debris and cell aggregates. The resulting sponge suspensions (C0) were centrifuged at increasing speeds (200, 500 and 10,000 g), following the protocol by Roué *et al.* [43], giving pellet fractions C1 (enriched in sponge cells), C2 (enriched in light sponge cells and some bacteria) and C3 (enriched in bacteria), respectively. Additionally, in order to detach surface-attached bacteria from *C. clathrus* sponge cells, fraction C1 was trypsinized for 15 min at 20°C (30 mg trypsin/g sponge wet weight), and the supernatant was centrifuged at 10,000 g to give fraction $C3_{tryp}$ (enriched in cell-surface associated bacteria). The protocol is illustrated in Fig. 2.

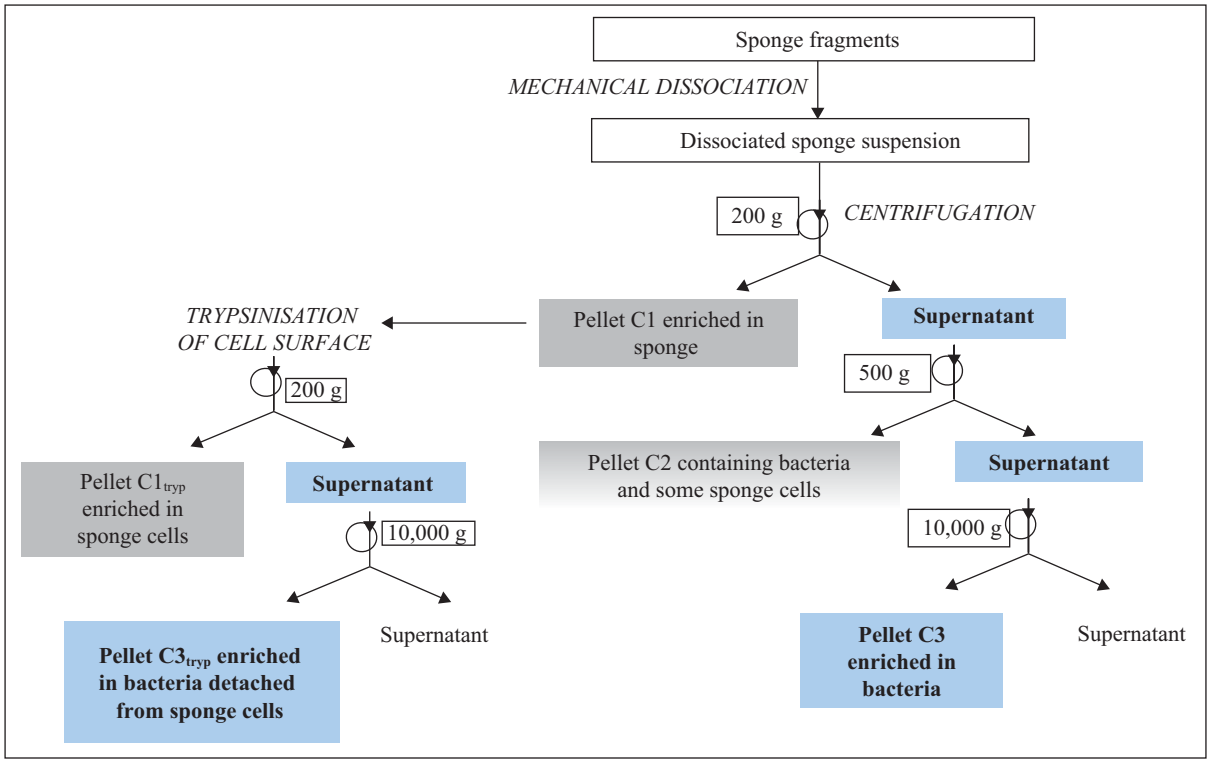


Fig. 2. Protocol for separation of bacterial or cell fractions from dissociated sponge *via* differential sedimentation.

5. Isolation of Heterotrophic Bacteria

For each sponge sample, the mechanically dissociated suspension (macerate) was diluted at 10^{-2} and 10^{-4} in 0.2 µm filtered seawater, and 100 µl of the corresponding dilutions were spread onto Marine Agar (Difco 2216 in 1.5% Agar, Fisher Scientific), which is a nutrient-rich generalist medium commonly used for marine bacteria. All the plates were incubated at 18°C under air and without light. After two days of incubation, colonies-forming units (CFU) were counted on the plates and their number standardized to the volume of the sponge maceration (CFU/mL) and the wet weight of the initial sponge fragment. Results are expressed as CFU/g wet weight. Morphologically different bacterial colonies were isolated and propagated in pure culture on Marine Agar.

6. Phylogenetic Affiliation of Cultivable Bacteria

Bacterial DNA was obtained from each strain isolated on Marine Agar in pure culture, by suspending colonies in 50 µL of sterile water, boiled for 5 min and centrifuged to pellet debris (14,000 g; 5 min). PCR amplification of 1200 bp length 16S rRNA gene was performed using the supernatant as DNA matrix and the universal bacterial primer set 27F (5′-GAGTTTGATCCTGGCTCA-3′) and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The reverse primer 1385R (5′-CGGTGTGTRCAAGGCCC-3′) was also used with the forward primer 27F in a first series of bacterial ribotype characterization (yielding slightly shorter sequences). PCR reactions (50 µL final volume) contained 1 µL of DNA strain, 1.25 U of Taq polymerase (VWR), 10 pmoles of forward and 10 pmoles of reverse primers (Eurofins MWG Operon), 2.5 µmol of each deoxynucleoside triphosphate (Invitrogen) in Taq polymerase buffer (VWR) diluted to 1X with sterile MilliQ water. Amplification reactions were conducted in a Mastercycler Personal PCR system (Eppendorf). The PCR steps included 94°C for 5 min, 32 cycles of 94°C for 1 min, 52°C for 30 sec, 72°C for 30 sec, and 72°C for 7 min. Amplified DNA was checked by 1.5% agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Quiagen). Purified 16S rRNA gene amplicons were directly sequenced in both directions by Cogenics/Beckman Coulter Genomics. Consensus sequences were compared with those in the 16S DNA databases EMBL Prokaryote or Genebank by using FASTA [36] to determine similarity with sequences of previously identified bacteria.

7. Bio-Guided Isolation of Sponge or Bacterial Metabolites with Antimicrobial Activity

Antimicrobial activities of bacterial isolates were tested in the soft agar diffusion assay, by spotting live cultures of the bacteria of interest in plates of soft agar (MB Difco 2216 in 0.75% Agar) seeded with reference pathogen bacteria *Staphylococcus aureus* ATCC 6538 or *Candida albicans* ATCC 66029.

8. Isolation of Parabens (1-9) from *Microbulbifer arenaceous* Related Strain L4n2

The CH_2Cl_2 extract of *Microbulbifer* L4-n2 was chroma-

tographed on a silicagel (Merck silica gel 70-230 mesh) column using cyclohexane with increasing amounts of ethyl acetate (AcOEt) as eluent. Bioassay-guided fractionation retained a maximum of activity in fractions eluted with 20% and 30% AcOEt. These fractions were subjected to reversed-phase HPLC column (C18 Uptisphere 250 × 7.8 mm) with increasing amount of acetonitrile in H_2O as eluent (flow rate: 1 mL. min^{-1}, wavelength: of 254 nm) to yield parabens **1-9** [40].

9. Chemical Investivation of the Sponge *Clathrina clathrus*

The 1:1 CH_2Cl_2/MeOH extract of *C. clathrus* was chromatographed on a C18 SPE column in a vacuum chamber (H_2O, H_2O/MeOH 1:3, MeOH, CH_2Cl_2, 100 mL of each). The fraction eluted with H_2O/MeOH 1:3 was subjected to semi-preparative reversed-phase HPLC (Gemini C6-phenyl 10 × 250 mm) with increasing amounts of CH_3CN/0.1% formic acid in H_2O/0.1% formic acid as eluent and afforded clathridimine **10**, clathridine **11**, and the zinc complex of clathridine **12** [43].

10. Isolation of Glycerolipids 13-14 from *Pseudoalteromonas citrea* Related Strain 1-Cc-11 and from the Sponge *Clathrina clathrus*

Representative strain 1-Cc-11 of *P. citrea* related bacteria, isolated from *C. clathrus* in November 2007, was grown in liquid Marine Broth Difco 2216 (37.4 g L^{-1}) under agitation 350 r.p.m. for 24 h in the dark at 15°C. At the end of exponential growth phase (DO ~1), cultures of strain 1-Cc-11 were centrifuged at 4°C for 15 min at 10,000 g. Supernatants were immediately poured into EtOAc 1:2 (v:v), for overnight extraction. Three separate 2 L cultures were pooled (total 6 L).

Chemical investigation by successive chromatographies of the 1-Cc-11 bacterial extract led to the isolation of several metabolites [43, 44], including the glycerolipids monopalmitin **13** (respectively 1.5 mg, 0.20%/L of 1-Cc-11 culture; and 1.2 mg, 0.03% of sponge dry weight) and monostearin **14** (respectively 1.4 mg, 0.23%/L of 1-Cc-11 culture; and 1.0 mg, 0.025% of sponge dry weight).

11. *In situ* Chemical Detection *via* Liquid Chromatography Coupled to Mass Spectrometry (LC/MS) of the Bacterial Metabolites in Extracts of the Sponges

The chemical signature of bacterial metabolites (i.e. parabens from *L. johnstoni* isolate *Microbulbifer* sp. L4n2, and glycerolipids from *C. clathrus* isolate *Pseudoalteromonas* sp. 1Cc-11) was searched in the mass spectra of crude extracts and separated bacterial fractions of the corresponding sponge, after LC with a protocol optimized for the purification of the molecules of interest. For example, in order to check the presence, in *C. clathrus* or in its derived fractions, of the compounds isolated from *P. citrea* related strain 1-Cc-11, LC/MS experiments were carried out on a micro/nano HPLC system (Ultimate 3000, dionex) equipped with a Jupiter column (C18, 159 × 1.0 mm, Phenomenex) and coupled with an API Q-STAR PULSARI (Applied Biosystem) apparatus. CH_2Cl_2/MeOH 1:1 extracts of lyophilized samples of *C. clathrus* (containing sponge cells and all sponge-associated resident bacteria) were compared with CH_2Cl_2/MeOH 1:1 extracts obtained by differential centrifugations of dissociated sponge suspensions. We previously used a similar approach to show that one of the three major antimicrobial 2-aminoimidazole alkaloids isolated from *C. clathrus* was localized in the sponge cells [43].

III. RESULTS AND DISCUSSION

1. *In situ* Visualization of Bacteria in Tissue Sections of Both Calcareous Sponge Models

Clathrina clathrus is a Mediterranean calcareous sponge belonging to the subclass Calcinea (family Clathrinidae), with low-density, thin mesohyl, and simple ascon organization of its aquiferous system (Fig. 3A-3D). By contrast, *Leuconia johnstoni* has also a thin mesohyl, but a more complex leuconoid organization of its aquiferous system, slowing the water flow through the sponge for increased particle retention efficiency (Fig. 3E-3H). Both sponges contain bacteria, visualized in tissue sections with Gram morphological staining of bacterial walls and by *in situ* hybridization with bacterial probe EUB338 (Fig. 3). Resident extracellular bacteria are located in the mesohyl gel and associated to the tissue layers lining external or internal surfaces (respectively pinacoderm and choanoderm/choanocyte chambers). Transient bacteria are also visible in the aquiferous canals (stars in Fig. 3H), due to the filtration by the sponge of environmental picoplankton for its nutrition. The spicular skeleton (calcium carbonate) of these sponges represents 35% of the dry weight of *C. clathrus* and 76% of the dry weight of *L. johnstoni*. This degree of mineralization influences the yield in metabolites which can be extracted from these sponges. Combined to their low biomass, this difficulty is a challenge which explains why chemical investigations of calcareous sponges are not frequent.

2. Attribution of the Sponge Antimicrobial Activity to Their Associated Bacteria or Their Dissociated Sponge Cell Fractions

Dichloromethane – Methanol (1:1) extract of *C. clathrus* exhibited antimicrobial activity against the yeast *C. albicans* and the Gram positive strain *S. aureus* (Table 1).

Dichloromethane extract of *L. johnstoni* exhibited a persistent annual antimicrobial activity against the Gram positive strain *S. aureus* (Table 1).

The activities of bacteria and cell enriched fractions were evaluated separately, in order to evaluate the contribution of the total associated bacterial community to the antimicrobial activity of each sponge. Indeed, most symbiotic microorganisms are uncultivable, and thus the putative microbial origin of natural compounds isolated from sponges is often inferred from indirect evidence, such as cellular localization data [41]. This method, independent of the microbiological culture, allows testing the contribution of the total bacterial

Table 1. Localization of the sponge biological activities (+ : active; -: inactive; ND: not determined).

Sponge	Extract (1 mg/disk)	Antimicrobial activity (inhibition diameter in mm)		
		S. aureus	*E. coli*	*C. albicans*
Clathrina clathrus	CH_2Cl_2/MeOH 1:1 crude extract	11 ± 2	15 ± 3	20 ± 1
	CH_2Cl_2/MeOH 1:1 extract of cell fraction	+	+	+
	CH_2Cl_2/MeOH 1:1 extract of bacterial fraction	-	-	+
Leuconia johnstoni	CH_2Cl_2 crude extract	11 ± 1	-	-
	CH_2Cl_2 extract of cell fraction	-	ND	ND
	CH_2Cl_2 extract of bacterial fraction	+	ND	ND
Control (commercial antimicrobials)	Kanamycine	37 ± 1	38 ± 1	-
	Itraconazole	-	-	21 ± 1

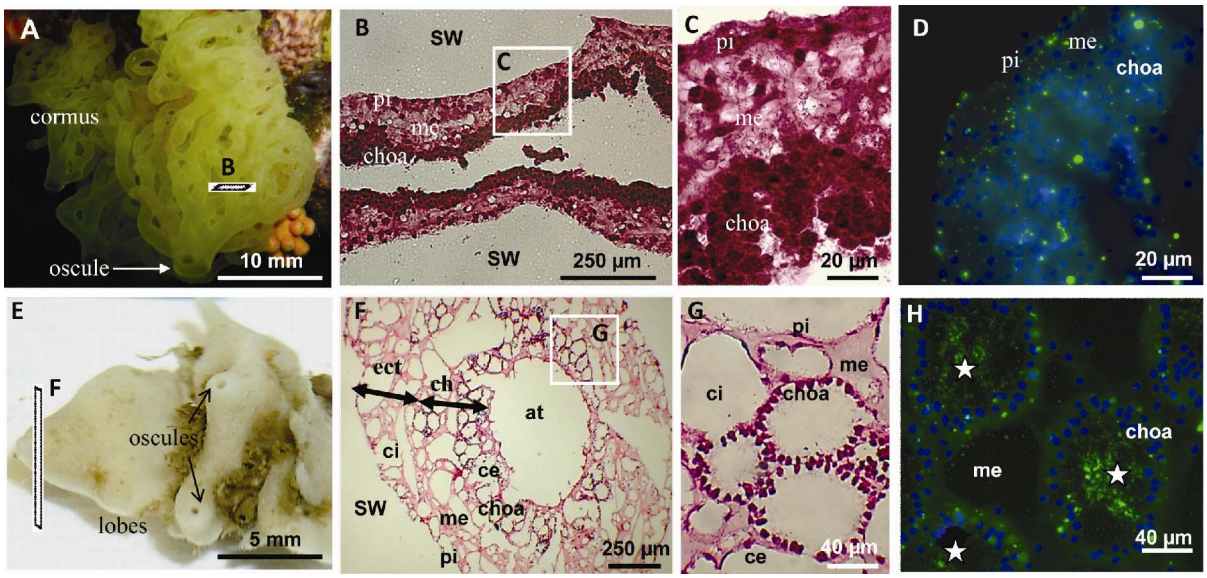


Fig. 3. Sponge tissue organization and localization of associated bacteria. A-D: *Clathrina clathrus* (A) morphology characterized by a cormus formed of anastomosed tubes with an oscule, (B) tissue section through tube showing the asconoid aquiferous system and inset (C) close-up of region in B. (D) Epifluorescence micrograph of tissue section through tube showing bacteria hybridized (CARD-FISH) with probe EUB338 (in green) and sponge cell nuclei counterstained with Dapi (in blue). E-H: *Leuconia johnstoni* (E) lobate morphology with several oscules at top of lobes, (F) tissue section through lobe showing the leuconoid aquiferous system, with the differentiation of an outer ectosome (ect) and an inner choanosome (ch), around the central atrial cavity (at); inset (G) close-up of region in F. (H) Epifluorescence micrograph of tissue section through the choanosome, showing bacteria hybridized (CARD-FISH) with probe EUB338 (in green) and sponge cell nuclei counterstained with Dapi (in blue). choa: choanoderm (*C. clathrus*) or choanocyte chamber (*L. johnstoni*), me: mesohyle, pi: pinacoderm, SW: seawater, ci: inhalant canal, ce: exhalant canal; asterisk: bacteria from seawater pumped into the choanocyte chambers for filtration.

community (cultivable plus uncultivable bacteria) to the chemical profile of its sponge host.

The respective CH_2Cl_2 extracts of sponge-derived fractions C1, C2 and C3 (containing increasing proportions of bacteria relative to sponge cells) showed different biological activities, depending on the sponge model species and on the selected reference strain. Either the microbial compartment of the sponge (in the case of *L. johnstoni* anti-*S. aureus* activity) or its cell fraction (in the case of *C. clathrus* antifungal activity) was responsible of the sponge biological activities (Table 1).

3. Phylogenetic Diversity of Cultivable Bacteria Isolated from the Sponges

After 2 days incubation at 18°C on nutrient-rich medium Marine Agar Difco 2216, the number of formed colonies ranged from $3 \pm 1 \times 10^5$ CFU g^{-1} wet weight in *C. clathrus* (6 replicates, covering all seasons) to $2.2 \pm 2 \times 10^6$ CFU g^{-1} wet weight in *L. johnstoni* (14 replicates, covering all seasons). In *L. johnstoni* the number of CFUs increased by a factor 10 between winter (lowest abundance) and fall (peak), with a smaller peak observed in spring. These seasonal differences

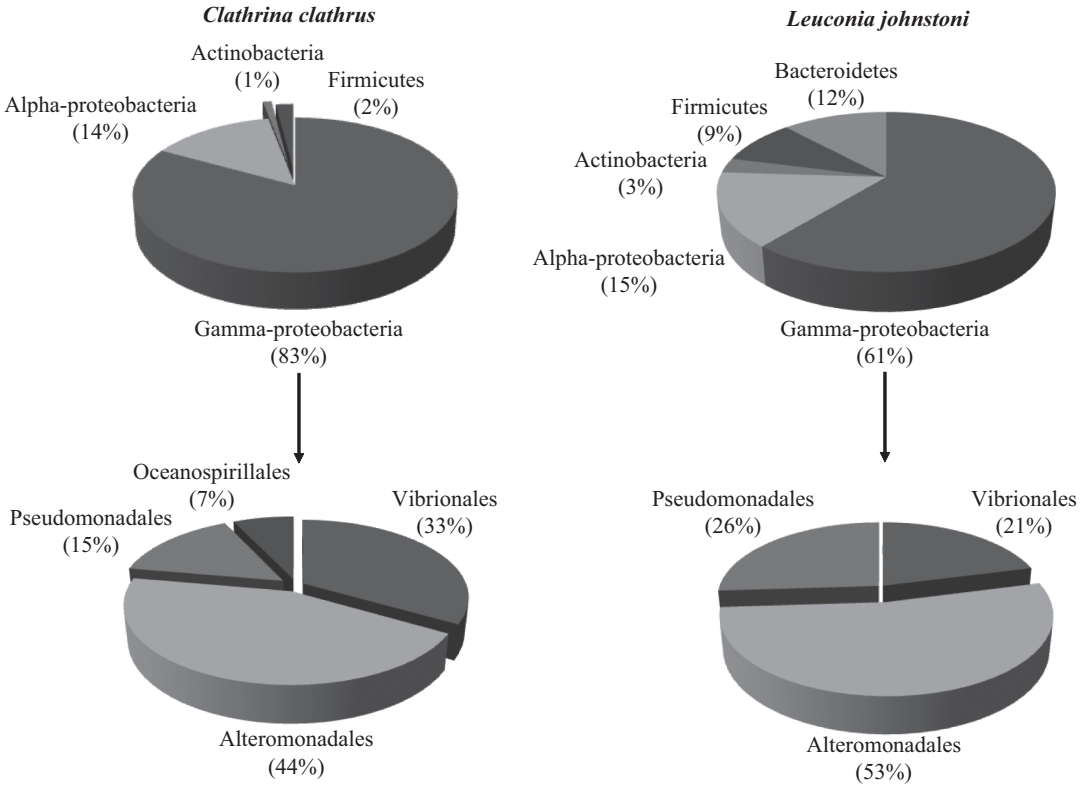


Fig. 4. Taxonomic diversity of cultivable bacteria associated with temperate calcareous sponges.

in bacterial abundance isolated from sponges could be related to seasonal changes in temperature and phytoplankton abundance in the marine environment, causing episodic bacterial blooms.

A total of 86 and 282 strains of heterotrophic aerobic bacteria were isolated respectively from *C. clathrus* and *L. johnstoni*, respectively. The taxonomic distribution of these cultivable bacteria (at phylum and order level) is illustrated in Fig. 4.

Taxonomic distribution of the isolates indicated a cultivable bacterial community with a similar structure in both calcareous sponge models (Fig. 4). This cultivable community was predominantly composed of Proteobacteria, with 60-80% Gamma-Proteobacteria, and about 15% Alpha-proteobacteria. Some Firmicutes (<10%), Actinobacteria (<5%) and Bacteroidetes (12% in *Leuconia johnstoni*) were also isolated. The majority of Gamma-proteobacteria was represented by Alteromonadales strains (about 50%), and Vibrionales strains (20-30%). This structure was maintained similar throughout the seasons. The recovered diversity depended on colony sampling effort, and results were qualitative, as colonies were selected for propagation in pure cultures based on their apparently different morphology on Marine Agar. This selection tended to introduce a bias for the selection of pigmented colonies. It is widely acknowledged that the cultivable fraction, isolated on generalist nutrient-rich media, corresponds to only about 0.1-1% of the total environmental bacteria [2]. Moreover, the obtained diversity of culturable bacteria may increase on oligotrophic microbiological media [48] or with a combination of specialized media.

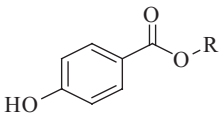
Bioguided screening of the antibacterial activity against *S. aureus* ATCC 6538 revealed 38 active strains for *L. johnstoni* (13.5%) and 9 active strains for *C. clathrus* (10.4%). Activity of these isolates could contribute to the antimicrobial activity initially observed in the whole sponge.

Among these bacterial isolates, we selected the most active strains, or the strains which were recurrently isolated, for further chemical investigations.

4. Natural Parabens Produced by *Microbulbifer* Strain L4n2 in Its Sponge Host *Leuconia johnstoni*

One of the most active strains isolated from *L. johnstoni*, named L4-n2, was extensively studied. The bacterial strain L4-n2 was phylogenetically affiliated with the genus *Microbulbifer* [40], family Alteromonadacae, Gamma-Proteobacteria sub-class, with 99.8% sequence homology with the 16S rRNA of *Microbulbifer arenaceous* [50]. *Microbulbifer* is a genus of Gamma-Proteobacteria found in seawater, in sediments [54] or associated with marine organisms such as sponges [37], ascidians [25], corals [32] or algae [35].

Examination of *L. johnstoni* sponge tissue sections, after fluorescent *in situ* hybridization with the specific probe MA445, confirmed the persistent presence of the strain L4-n2, with a positive signal observed in the four seasons [40].



1 R = $-CH_2-CH_3$
2 R = $-(CH_2)_3-CH_3$
3 R = $-(CH_2)_7-CH_3$
4 R = $-(CH_2)_9-CH_3$
5 R = $-(CH_2)_2-CH(OH)-(CH_2)_6-CH_3$
6 R = $-(CH_2)_8-CH(CH_3)_2$
7 R = $-(CH_2)_2-CH(OH)-(CH_2)_5-CH(CH_3)_2$
8 R = $-(CH_2)_{11}-CH_3$
9 R = $-(CH_2)_4-CH=CH-(CH_2)_5-CH_3$

Fig. 5. Antimicrobial esters of para-hydroxybenzoate from *L. johnstoni*.

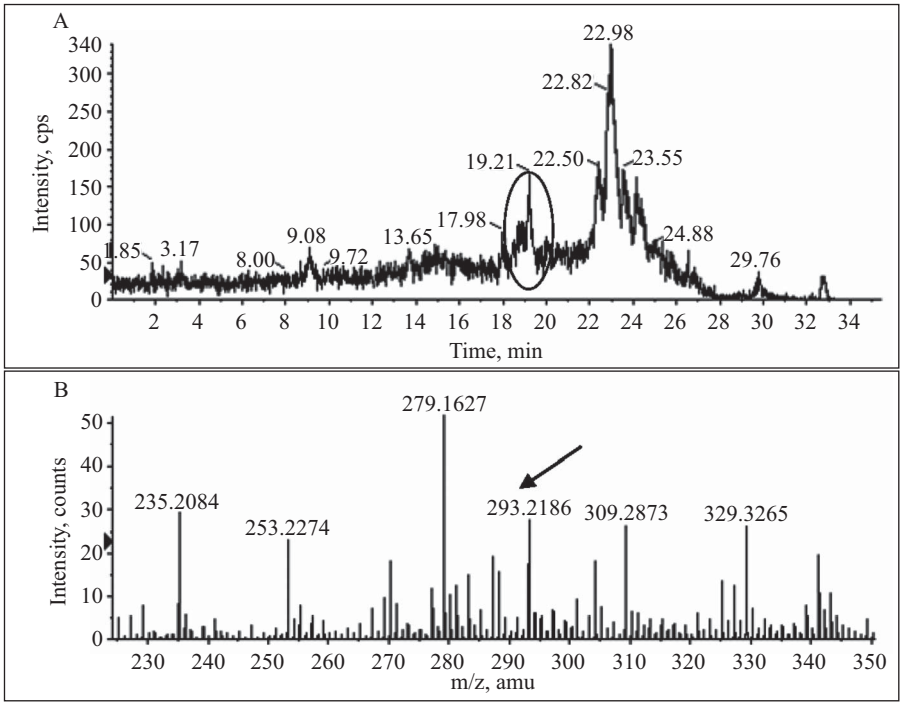


Fig. 6. Detection of methyldecylparaben by LC/MS in the CH_2Cl_2 extract of *L. johnstoni* (January 2006). A: HPLC profile total ion current chromatogram selecting for retention time: 18 minutes. B: Positive ESI mass spectrum revealing the presence of methyldecylparaben.

In order to identify chemical mediators which inhibited the growth of *S. aureus* ATCC 6538, a bioguided fractionation of the CH_2Cl_2 crude extract of the *Microbulbifer* L4-n2 culture was performed. Successive chromatographies of the CH_2Cl_2 crude extract on silica gel and reverse-phase columns, guided by antimicrobial bioassays, afforded nine compounds belonging to the paraben series. In addition to ethyl and butylbenzoates **1-2,** already known as synthetic compounds**,** seven natural analogues **3**-**9** were isolated, including the four new natural parabens **5**, **6**, **7** and **9** [40]. Their structures were determined by mass and 2D NMR spectral analysis (Fig. 5). Compounds **1**, **3**, **4**, **8** were reported for the first time as natural products. These compounds were known as preservatives in pharmaceutics and cosmetics.

These nine esters of para-hydroxybenzoic acid isolated from *Microbulbifer* sp. L4-n2 were identified as responsible of the anti-*S. aureus* activity. All of them exhibited antimicrobial activity assessed as values of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) [40].

Compound **4** exhibited the greatest efficiency against *S. aureus* with MIC values of 2.8-5.6 µM. The length of the alkyl chain seemed to increase considerably activity. We found that compounds **3**, **4**, **6**, **8**, which bear a C-8, C-10, C-11 and C-12 alkyl chain, respectively, revealed a higher potency and appeared about 100 fold more active than methyl and propyl synthetic parabens as well as ethyl and butyl natural parabens (**1**-**2**). The presence of a hydroxyl functionality on the alkyl chain had little effect upon antimicrobial activity.

These para-hydroxybenzoate esters were also detected, by Liquid Chromatography/Mass Spectrometry (LC/MS), in the different CH_2Cl_2 extracts of the sponge at every season (Fig. 6, illustrating a profile obtained for January 2006). These results indicate a persistent annual production of these bacterial metabolites within the sponge host, and suggest their contribution to the permanent anti-*S. aureus* antimicrobial activity of *L. johnstoni*.

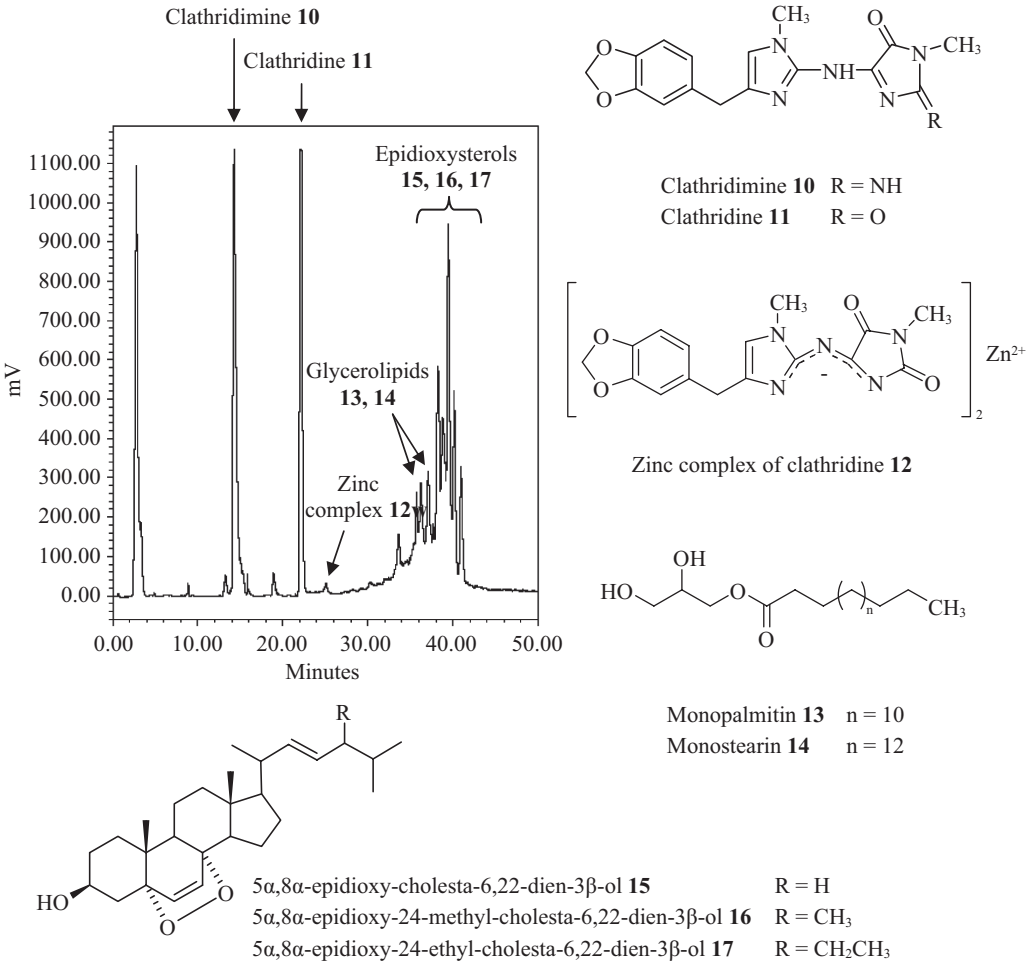


Fig. 7. HPLC/ELSD chromatogram of the CH_2Cl_2/MeOH 1:1 extract of the sponge *Clathrina clathrus* and structures of isolated secondary metabolites belonging to three chemical families: 2-aminoimidazole alkaloids (10-12), glycerolipids (13-14) and epidioxysterols (15-17).

Bacterial antagonistic interactions were studied between *Microbulbifer* sp. strain L4-n2 (Gamma-proteobacteria) and an environmental, Gram-positive, *Bacillus* sp. strain L6-3e (Firmicutes), co-isolated from the same sponge. In addition to their anti-*S. aureus* activity, the para-hydroxybenzoic acid esters from *Microbulbifer* L4-n2 were also found to be responsible for growth-inhibition of this *Bacillus* sp. strain L6-3e [40]. This result identifies the paraben metabolites of *Microbulbifer* as chemical mediators involved in the competition between sponge bacterial isolates.

5. Recurrent Isolation from the Sponge *C. clathrus* of Biologically Active *Pseudoalteromonas* sp. Bacteria Affiliated to *P. citrea*

In *C. clathrus*, about 4% of all strains in the cultivable bacterial community shared similar morphology, i.e. bright yellow-pigmented, circular, regular and smooth colonies. Such colonies were recurrently isolated in four consecutive years (2007-2010) from specimens collected in winter, spring, summer and fall, in the same northwestern Mediterranean geographical site off Marseille. They were obtained from the mechanically dissociated C0 sponge suspensions (3 strains from 2 specimens), as well as from the derived C1 heavy fraction enriched in sponge cells (5 strains from 3 specimens), its supernatant fraction C5 obtained after trypsinization of the sponge cell surface (2 strains from 1 specimen), and from the light fraction C3 enriched in bacteria (1 strain from 1 specimen).

These yellow-pigmented strains were characterized by a similar Gram-negative thin rod morphotype. They displayed a similar broad spectrum of antimicrobial activities, when cultured at 20°C in soft (0.75%) Marine Agar, against the bacterium *S. aureus* ATCC 6538 and the yeast *Candida albicans* ATCC 66029.

Based on their partial 16S rRNA sequence (1,200 bp), the bright yellow pigmented strains were affiliated with the genus *Pseudoalteromonas* (Gamma-Proteobacteria, Alteromonadales) (n = 9 sequences obtained from a total of 22 representative colonies, isolated throughout the total sampling time-series). Their 16S rRNA gene sequences shared more than 99.5% sequence similarity with *Pseudoalteromonas citrea* NCIMB 18T and *Pseudoalteromonas aurantia* ATCC 33046T, both

previously isolated from the northwestern Mediterranean surface seawater. Their isolation at every season in several samples of *C. clathrus* suggests persistent interaction of these environmental bacteria with this calcareous sponge.

6. Chemical Study of the Calcisponge *C. clathrus*

The chemical investigation of the CH_2Cl_2/MeOH 1:1 extract of the sponge *C. clathrus* led to the isolation of eight metabolites belonging to three different chemical families (Fig. 7): three 2-aminoimidazole alkaloids (the new clathridimine **10** along with the known clathridine **11** and its zinc complex **12**), two glycerolipids (monopalmitin **13** and monostearin **14**) and three epidioxysterols (5α,8α-epidioxy-cholesta-6,22-dien-3β-ol **15**, 5α,8α-epidioxy-24-methyl-cholesta-6,22-dien-3β-ol **16** and 5α,8α-epidioxy-24-ethyl-cholesta-6,22-dien-3β-ol **17**) [44]. The new clathridimine **10** differed from the known clathridine **11** only by the replacement of a carbonyl by an imino group at the C-9 position. Its structure was solved by comparison of NMR data with those of other 2-aminoimidazole alkaloids sharing a similar structural modification [43]. Clathridine **11** and its zinc complex **12** were previously isolated from *C. clathrus* [8, 9] whereas the glycerolipids and the epidioxysterols were isolated for the first time from *C. clathrus* and more generally from a calcareous sponge. However, the glycerolipids were previously detected in other marine organisms [7, 34, 39] and the epidioxysterols isolated from several demosponges [19, 20, 28, 29].

The CH_2Cl_2/MeOH 1:1 extract of *C. clathrus* showed a broad spectrum of activities against the bacterial strains *S. aureus* and *E. coli* and the yeast *C. albicans*. All the eight compounds isolated from *C. clathrus* were tested against these three microorganisms and only the three 2-aminoimidazole alkaloids **10-12** revealed activities [43]. Clathridine **11** displayed antifungal activity against *C. albicans* whereas its zinc complex **12** exhibited anti-*S. aureus* activity. The antifungal activity of clathridine **11** had been previously described with inhibition of growth of *C. albicans* and *Saccharomyces cerevisiae* [8]. The new clathridimine **10** inhibited growth of the bacterial pathogen *E. coli* and the fungal pathogen *C. albicans*. Then, a simple modification of a carbonyl into an imine confers an antibacterial activity to the clathridimine **10**. These results might suggest a role for these compounds as antimicrobial chemical defense of *C. clathrus*.

In order to determine the cellular localization of secondary metabolites isolated from the sponge *C. clathrus* (e.g. 2-aminoimidazole alkaloids **10-12**, glycerolipids **13-14** and epidioxysterols **15-17**), sponge cells were separated from bacteria through differential centrifugations of dissociated sponge suspensions. Moreover, trypsinization of sponge cell surface was conducted in order to improve separation of sponge cells from extracellular bacteria which may be closely associated to the sponge cell surface [43]. The CH_2Cl_2/MeOH 1:1 extracts of lyophilized samples of *C. clathrus* (containing sponge cells and all sponge-associated resident bacteria) were compared with CH_2Cl_2/MeOH 1:1 extracts obtained by differential centrifugations of dissociated sponge suspensions.

HPLC/UV/DEDL profiles and LC/MS analyses of CH_2Cl_2/MeOH 1:1 extracts of fractions enriched either in sponge cells or in bacteria allowed localization of the clathridine **11** and its zinc complex **12** in the sponge-derived fractions C1 and $C1_{tryp}$, i.e. in the cells of *C. clathrus* [43]. These results suggest the production of these two antimicrobial 2-aminoimidazole alkaloids by the sponge and not by extracellular associated bacteria. The new clathridimine **10** could not be localized because of its instability in water.

The results obtained with this approach for glycerolipids **13-14** and epidioxysterols **15-17** were too ambiguous to conclude. Indeed, the glycerolipids **13-14** and epidioxysterols **15-17** were detected by LC/MS both in the sponge cell-enriched C1 heavy fraction (sponge cells, mostly choanocytes) and in the bacteria-enriched C3 light fraction (containing all resident bacteria). Thus the results of the differential sedimentation experiments could not determine conclusively a sponge or bacterial origin of these metabolites.

7. Chemical Study of Cultures of *Pseudoalteromonas* Strain 1-Cc-11

Chemical investigation (HPLC/UV/DEDL profiles and LC/MS analyses of CH_2Cl_2/MeOH 1:1 extracts) of pure cultures of one representative of these bright yellow pigmented *Pseudoalteromonas* sp. bacteria, the *P. citrea* strain 1-Cc-11, showed the absence of antimicrobial 2-aminoimidazole alkaloids **10-12** previously isolated from *C. clathrus* but suggested the presence of monopalmitin **13** and monostearin **14** (Fig. 8). Purification of the CH_2Cl_2/MeOH 1:1 extract of culture broth was then conducted in order to isolate these glycerolipids. Monopalmitin **13** and monostearin **14** were purified from 1-Cc-11 cultures in MB Difco 2216, respectively at 0.20%/L and 0.23%/L, confirming a bacterial production of these glycerolipids. Both these compounds had also been previously isolated from the whole sponge. Based on the phylogenetic (16S rRNA) affiliation of *P. citrea* strain 1-Cc-11 to environmental seawater bacteria, it is possible that these *P. citrea* are food bacteria filtered by the calcareous sponge *C. clathrus* and contributing with glycerolipids monopalmitin **13** and monostearin **14** to the carbon nutrition of the sponge. However, these glycerolipids are simple metabolites with a membrane structural function. Their isolation in parallel in extracts of bacterial cultures of a *P. citrea* strain and in extracts of sponge tissue does not imply that *P. citrea* bacteria are the sole source of glycerolipids in the sponge. These compounds may be produced by the sponge itself, or provided by other bacteria, archea and picoeukaryotes, either resident in the sponge or filtered by the sponge for its nutrition. The difficulty to conclude about the origin of these glycerolipids is due to their simple structure and ubiquitous production by many possible prokaryotic and eukaryotic sources.

Five other compounds were also isolated from the CH_2Cl_2/MeOH 1:1 extracts of pure cultures of the strain 1-Cc-11: three diketopiperazines (Cyclo-*L*-Pro-Gly **18**, Cyclo-[(*trans*-*L*-hydroxy-Pro)-*L*-Leu] **19**, Cyclo-[(*trans*-*L*-hydroxy-Pro)-*L*-Phe]

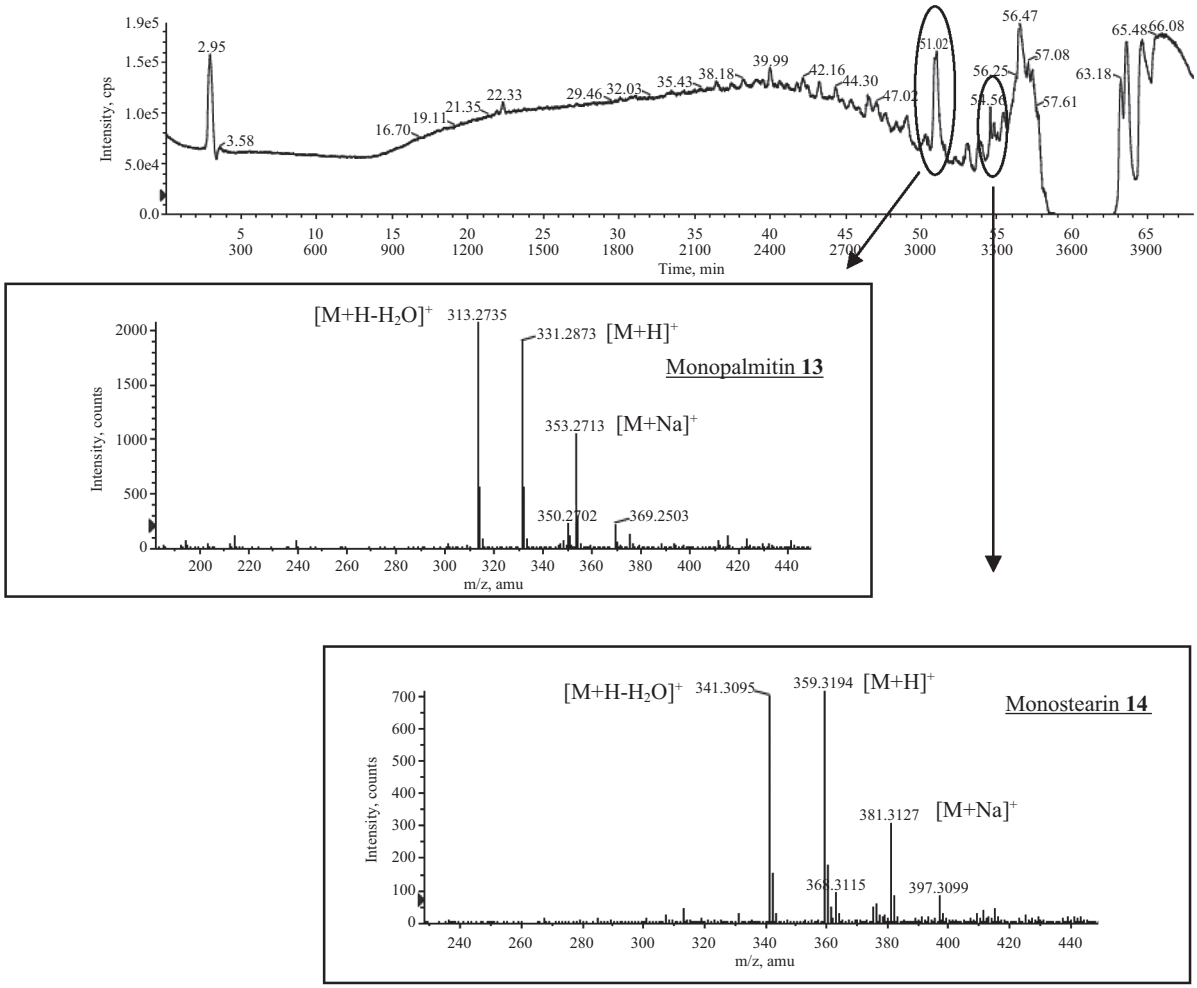


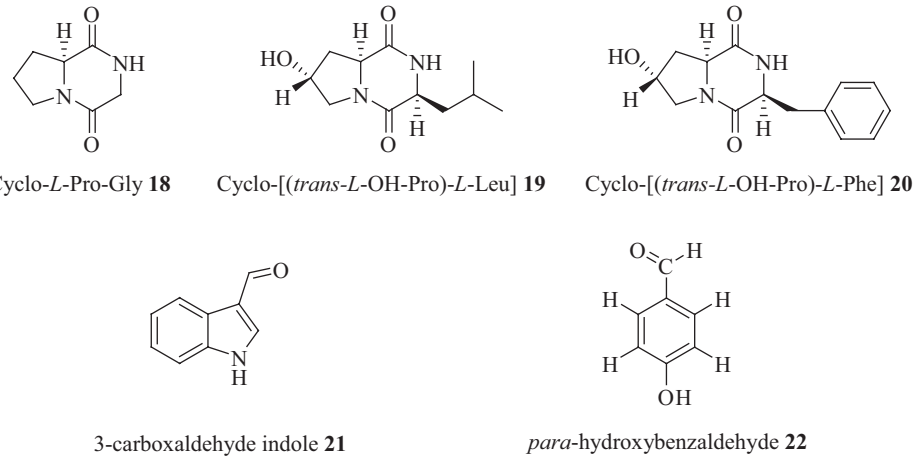
Fig. 8. Chemical signature (obtained by LC-MS analyses) of glycerolipids 13 and 14 (previously isolated from *Clathrina clathrus*) in the CH_2Cl_2/MeOH 1:1 extract of *Pseudoalteromonas* sp. strain 1-Cc-11 indicating their presence in the pure culture and thus their bacterial production. HPLC profile total ion current chromatogram of the CH_2Cl_2/MeOH 1:1 extract of *Pseudoalteromonas* sp. strain 1-Cc-11 and positive ESI mass spectrum at retention time 51.0 and 54.6 respectively revealing the presence of glycerolipids 13 and 14.

20), an indole (3-carboxaldehyde indole **21**), a benzenic derivative (*para*-hydroxybenzaldehyde **22**) (Fig. 9) [44]. However, none of these compounds **18-22** were responsible for the antimicrobial activities against *S. aureus*, *C. albicans* and *V. splendidus* of the bright yellow pigmented *Pseudoalteromonas* sp. bacteria strains. Diketopiperazines **18-20** were previously isolated from bacteria associated to marine organisms [10, 12, 24, 27] as well as compound **22** [3].

IV. CONCLUSION

Assessing the contribution of the associated bacterial community to the chemical diversity of calcareous sponges has been approached by combined culture-dependent and independent methods, using *Leuconia johnstoni* and *Clathrina clathrus* as biological models.

Antimicrobial activity of *L. johnstoni* was localized in the bacterial fractions through differential sedimentation, leading to the identification of paraben compounds produced by the low abundance but persistent sponge resident bacteria *Microbulbifer* sp. cf. *arenaceous* L4n2. These bacterial parabens included some new structures very active against *S. aureus*. On the opposite, the antifungal activity of *C. clathrus* was identified in both cellular and bacterial compartments. Among the eight isolated compounds, two of them, clathridine and its zinc complex, were detected in the sponge cell enriched fraction, suggesting their production by the sponge itself. Bacteria affiliated to the genus *Pseudoalteromonas* and having the same spectrum of activity that *C. clathrus* were repeatedly isolated. Chemical study of a representative strain led to the isolation of seven natural products, among which the compounds monopalmitin and monostearin were previously isolated from the whole sponge. This finding suggests a *Pseudoalteromonas* bacterial origin of these two compounds. However, given their ubiquitous nature, it is not excluded that other bacteria or microorganisms could provide these compounds to *C. clathrus*.



Cyclo-*L*-Pro-Gly **18** Cyclo-[(*trans*-*L*-OH-Pro)-*L*-Leu] **19** Cyclo-[(*trans*-*L*-OH-Pro)-*L*-Phe] **20**

3-carboxaldehyde indole **21** *para*-hydroxybenzaldehyde **22**

Fig. 9. Structures of secondary metabolites 18-22 isolated from the CH_2Cl_2/MeOH 1:1 extract of the *Pseudoalteromonas* sp. 1-Cc-11 strain together with monopalmitin 13 and monostearin 14 which were also previously isolated from *C. clathrus*.

These cellular localization studies demonstrate that the origin of the metabolites isolated from sponges is a very complex issue and must be considered carefully for each metabolite and each sponge species. Results obtained through this study contribute to a better knowledge of the chemical diversity of calcareous sponges and of the cultivable bacterial diversity that they host. The illustrated methods can be applied in the future to study chemical mediators involved in the complexity of bacteria-bacteria interactions encountered in sponge/bacteria associations.

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