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Sponging up metals: Bacteria associated with the marine sponge *Spongia officinalis*

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ABSTRACT

The present study explored the bacteria of the sponge *Spongia officinalis* in a metal-polluted environment, using PCR-DGGE fingerprinting, culture-dependent approaches and *in situ* hybridization. The sponge samples collected over three consecutive years in the Western Mediterranean Sea contained high concentrations of zinc, nickel, lead and copper determined by ICP-MS. DGGE signatures indicated a sponge specific bacterial association and suggested spatial and temporal variations. The bacterial culturable fraction associated with *S. officinalis* and tolerant to heavy metals was isolated using metal-enriched microbiological media. The obtained 63 aerobic strains were phylogenetically affiliated to the phyla Proteobacteria, Actinobacteria, and Firmicutes. All isolates showed high tolerances to the selected heavy metals. The predominant genus *Pseudovibrio* was localized *via* CARD-FISH in the sponge surface tissue and validated as a sponge-associated epibiont. This study is the first step in understanding the potential involvement of the associated bacteria in sponge's tolerance to heavy metals.

1. Introduction

Marine sponges are sessile filter feeders that pump actively the surrounding seawater, feed on suspended organic material and pico and nanophytoplankton (Topçu et al., 2010) and can accumulate high amounts of metals according to the contamination of the environment (Cebrian et al., 2003; Hansen et al., 1995). They have thus been proposed as sentinels of heavy metal pollution (De Mestre et al., 2012; Patel et al., 1985; Pérez et al., 2005).

Marine sponges are known to harbor within their tissues dense and diverse communities of microorganisms such as fungi, microalgae, archaea and bacteria, which can constitute up to 40% of the volume of some *Demospongiae* (Hentschel et al., 2006). Although little is known about the role of these microorganisms for their host, it was proposed that they contribute to the sponge metabolism as a source of nutrition (Webster and Taylor, 2012; Wilkinson and Garrone, 1980) and to its defense through the production of secondary metabolites against predators, competitors, fouling organisms or microbes (Bakus et al., 1986; Taylor et al., 2007). Resistance to heavy metal has been described for numerous environmental bacteria isolated from seawater (Sabry et al., 1997), corals (Sabdono et al., 2012), polychaete annelids (Jeanthon and Prieur, 1990) or recently from the Brazilian sponge *Polymastia janeirensis* (Santos-Gandelman et al., 2014). Bacterial resistance to heavy metals relies on a large variety of mechanisms

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including membrane transport, metal sequestration or enzymatic detoxification systems (Bruins et al., 2000; Nies, 1999). Several investigations have shown that the presence of heavy metals in the environment, due to pollution or anthropogenic activities, could affect bacterial communities and their activities (Gans et al., 2005; Losfeld et al., 2014). If the capacity of marine sponges to accumulate large amounts of heavy metals is well documented (Cebrian et al., 2003; Mayzel et al., 2014; Pan et al., 2011), very few studies investigate the relationships between putative changes in their associated microbial communities composition and the presence of heavy metals in the environment. The sponge *Rhopaloeides odorabile* was reported to show a decrease in density and diversity of its total bacterial community when exposed to cupric ion, with the selection of bacteria highly tolerant to copper (Webster et al., 2001). For the sponge *Fasciospongia cavernosa*, the associated culturable bacteria, affiliated to *Streptomyces* sp., *Salinobacter* sp., *Micromonospora* sp., *Roseobacter* sp., *Pseudomonas* sp., *Vibrio* sp., *Saccharomonospora* sp. and *Alteromonas* sp., were presented as suitable indicators of heavy metal contaminations (Selvin et al., 2009). Finally, specific variations in bacterial community composition were proposed to be responsible for differential accumulation patterns of metals between Red Sea sponges (Pan et al., 2011). Hence, sponge-associated microbial communities have been proposed as sensitive bioindicators of the heavy metal contamination of a marine habitat and may be partly responsible for metal accumulation within the sponge.

The Mediterranean *Spongia officinalis* Linnaeus, 1759, one of the commercial bath sponges, is abundant in the Provence littoral region, which is impacted by the influence of several sewages effluents including that of the city of Marseille, located in the Cortiou cove and a well-known source of anthropogenic pollutants (Pérez et al., 2005). *S. officinalis* has been used for its potential capacity to strongly concentrate all the trace metals and thus to reflect metal availability in the ecosystem (Pérez et al., 2004; Rainbow, 1995). This capacity was illustrated with the monitoring of the availability of twelve metals in nine sites near the city of Marseille after the start of its first sewage treatment plant (Pérez et al., 2005). *S. officinalis* revealed high content in all trace metals except for mercury and cadmium, and all metallic concentrations subsequently decreased after the opening of the treatment plant.

The aim of the present study was to investigate the bacterial community associated with *S. officinalis* and tolerant to heavy metals. We first determined with Inductively Coupled Plasma Mass Spectrometry (ICP-MS) the level of metallic contamination of *S. officinalis* and seawater samples from two Mediterranean sites, Cortiou and Riou, over three years. Secondly, we fingerprinted with Denaturing Gradient Gel Electrophoresis (PCR-DGGE) the diversity of the sponge- and seawater-associated bacterial community, which was analyzed according to metal concentrations both in sponge and surrounding seawater samples. Finally, we focused our investigations on the culturable bacterial community associated with *S. officinalis* for its tolerance to selected heavy metals. The isolated strains were phylogenetically affiliated and the *Pseudovibrio* genus was localized *in situ* with Catalyzed amplified reporter deposition fluorescence *in situ* hybridization (CARD-FISH) in the sponge tissue.

2. Materials and methods

2.1. Sponge and seawater sampling

Specimens of *S. officinalis* (*Demospongiae*, *Dictyoceratida*, *Spongiidae*) were collected by scuba diving at Cortiou and Riou (France) at 10 and 18 m of depth, respectively, in October 2011, September 2012 and December 2013 (Supporting Information, Figure S1). Cortiou, located 300 m east of the discharge outlet in the vicinity of the city of Marseille, is known to be strongly influenced by sewage from Marseille and its suburb (Pérez et al., 2005). Surrounding seawater samples were either directly frozen for metal analysis (2 × 50 mL per site and per year) or filtered on a 0.2 μm polycarbonate membrane (Millipore, 47 mm for microbiological analyses, 2 × 1 L per site and per year). Filter membranes, sponge samples and unfiltered seawater samples were stored at −80 °C.

2.2. Treatment of the samples for metal analysis

For each sampling, five freshly collected *S. officinalis* samples and two unfiltered seawater samples were collected from each site in October 2011, September 2012 and December 2013. *S. officinalis* samples were then lyophilized. The samples were prepared for metal analysis as follows: seawater samples were diluted tenfold in a solution containing 2% nitric acid in ultrapure water (18 MΩ) and sponges were mineralized in nitric acid (Sigma Aldrich, puriss. P.a., 65%), by the way of a microwave system. The ICP-MS instrument was a Thermo X series II model, equipped with a collision cell. The calibration curve was obtained by dilution of a certified multi-element solution (SCP Sciences). Metal concentrations were determined using Plasmalab software (Thermo-Electron, Les Ulis, France). The results for sponge samples, given as means and standard deviations expressed in μg g^{-1} of dry weight (DW), were analyzed statistically for differences between the years and sites by the Pairwise-Wilcoxon rank sum test using Bonferroni. Dry weights were transformed as sponge volumes by the water-dipping method (Stimson and Kinzie, 1991) in order to calculate the metal accumulation factors by dividing metal concentrations in sponge (μg L^{-1}) by metal concentrations in seawater (μg L^{-1}). Accumulation factors were analyzed statistically for differences between sites by the Wilcoxon test.

2.3. Total DNA extraction from sponge and seawater samples

Total DNA was extracted from *S. officinalis* after a protocol derived from two publications (Lee et al., 2009; Zhou et al., 1996). Two duplicates of 400 mg sponge tissues from Riou and Cortiou (October 2011, September 2012 and December 2013) were disrupted by a TissueLyser II (QUIAGEN). Samples were mixed with 400 μL extraction buffer (100 mM Tris–HCl, 100 mM EDTA, 100 mM NaPPi, 1.5 M NaCl, 27.4 mM cetrimonium bromide, pH 8.0) and incubated with proteinase K at a final concentration of 1 mg mL^{-1} at 37 °C for 30 min with regular inversions every 5 min. A second treatment was performed with 720 μL of 0.7 M sodium dodecyl sulfate (SDS) and incubated at 65 °C for 2 h with gentle inversions every 20 min. The supernatants obtained after centrifugation at 13,000 g at room temperature for 10 min were collected and transferred into 2 mL clean tubes. The pellets were extracted with 200 μL extraction buffer and 20 μL 0.7 M SDS, then vortexed for 10 s, incubated at 65 °C for 10 min and centrifuged at 14,000 g at room temperature for 10 min. Purification of total DNA of the combined supernatants was performed with 660 μL of the mixture phenol: chloroform: isoamyl alcohol (25:24:1) and vortexed for 20 s. After centrifugation at 14,000 g for 10 min, 0.6% (v/v) isopropanol was added to the aqueous phase and incubated at room temperature for 1 h. The supernatant was separated from the pellet by centrifugation at 14,000 g for 20 min at room temperature and the DNA-containing pellet was washed with 100 μL of a cold solution of 70% ethanol, covered with 50 μL of sterile deionized water and stored at −20 °C.

Filter membranes (0.2 μm) of the seawater samples (2 L) were aseptically cut into eight pieces and the DNA was extracted following the method described previously.