

Reduction of RBL-2H3 cells degranulation by nitroaromatic compounds from a *Bacillus* strain associated to the Amazonian sponge *Metania reticulata*

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Symbionts in sponges must interact with the host immune system, and this can be mediated by immunomodulators. As the bases of the immune system in sponges resemble those of higher metazoans, it is possible that compounds from this microbiota show similar effects in other phyla. It is also known that several antibiotics, in special macrolides, can modulate many components of the immune response and sponges and their associated microorganisms are a rich source of these compounds. Therefore, we tested the immunosuppressive capacity of antibiotic substances produced by bacterial and fungal strains isolated from the Amazon freshwater sponge Metania reticulata. Fourteen bacterial and six fungal strains were obtained from samples of M. reticulata collected in the Negro River (Amazon Central Basin region), during the dry season. These cultures were monitored for natural antimicrobial activity, and two Bacillus strains (MERETb.761 and MERETb.762) and one fungus (MERETf.010) were selected. One Bacillus strain, MERETb.762, showed strong and specific antibiosis on Staphylococcus aureus and two fractions of its extract inhibited the degranulation of RBL-2H3 cells. The predicted formulas of these fractions were C₁₂H₆N₄O₈ and C₂₅H₄N₂O₆, both corresponding to nitroaromatic compounds.

Keywords: Porifera, symbiosis, antibiosis, immunosuppressants

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INTRODUCTION

Sponges (phylum Porifera) represent one of the oldest extant multicellular animals (Müller, 1998), inhabiting a variety of marine and freshwater ecosystems. They are sessile filter-feeding organisms with a relatively simple body, constituted by an outer epithelial layer (pinacocytes) enclosing the mesohyl, formed by specialized cells, extracellular matrix and a network of canals and chambers. The water is pumped continuously throughout those chambers by flagellated cells (choanocytes) that retain and phagocytize the suspended particles (Simpson, 1984). Despite the absence of conspicuous physical defences, such as shells or spines, sponges are found in environments where the competition by substrate and predation are extremely aggressive (Ruzicka & Gleason, 2008; Turon *et al.*, 2009). This success is probably due to a wide variety of bioactive compounds, which have turned these animals into one of the most prolific sources of natural products (see Blunt *et al.*, 2014, and previous reviews of this series). However, many of these substances

are not produced by the sponge itself, but by its associated microbiota (Costantino *et al.*, 1999; Sipkema *et al.*, 2011). Recently there has been a trend to isolate, cultivate and identify sponge-associated microorganisms to search for new compounds and to obtain active substances in larger quantities (Taylor *et al.*, 2007; Schippers *et al.*, 2012).

Sponges are known to harbour a species-specific microbiota, which is maintained under strict control in healthy animals (Lee *et al.*, 2001; Jadulco *et al.*, 2002; Webster *et al.*, 2004). In addition, sponges are also exposed to large numbers of microorganisms from the surrounding environment (Pfannkuchen *et al.*, 2009). Sponges filter vast quantities of water, retaining more than 80% of the suspended particles. Therefore, a transient microbiota is always present in its channels, tissues and surfaces (Pile *et al.*, 1996). To deal with these environment-derived microorganisms, they present a well-organized innate immune system, which represents an efficient first line of defence and allows immediate responses using molecules that recognize highly conserved microbial structures (Wiens *et al.*, 2007). Recently, it has been shown that the bases of this system resemble those of higher metazoans (Müller *et al.*, 1999, 2002). The immune reactions in sponges are affected by suppressants such as FK506 and Cyclosporine A (CsA), which are produced by microorganisms and widely used in human organ transplants (Müller *et al.*, 2001; Sabella

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et al., 2007). This fact suggests that at least part of the symbiotic microbiota may be using a similar strategy to modulate the sponge immune system and avoid elimination.

It is known that several antibiotics, in special macrolides, are able to modulate many components of the immune response (Tamaoki, 2004; Altenburg *et al.*, 2011). Consequently, antimicrobial screening can indicate the presence of such compounds. In view of this, and the fact that many bioactive molecules detected in sponges have antibacterial properties, we tested the capacity of these substances isolated from sponge-associated microbionts to inhibit the degranulation of RBL-2H3 cells. This cell line, like mast cells and basophils, respond with degranulation as a consequence of immunological and non-immunological stimulation (Narenjkar *et al.*, 2006; Passante & Frankish, 2009). Substances that are able to block this process may be potential immunosuppressive molecules.

MATERIALS AND METHODS

Sponges

Samples of *Metania reticulata* (Bowerbank, 1863) (Haplosclerida: Metaniidae) were collected in the Negro River, in the Brazilian Amazon Central Basin region (Manaus, Amazon state, Brazil) (Figure 1), during the dry season (January). The sponges, including gemmules, were attached to trees positioned 10 m from the river margins and 1 m from the ground. The samples were collected with forceps, placed in sterile tubes and maintained at 8°C until processed.

Microbial isolation and culture

The external portions of the sponges were carefully removed with sterile scalpels and pieces (1 cm³) were collected from



Fig. 1. The freshwater sponge *Metania reticulata* attached to vertical tree branches in the Negro river during the dry season.

the interior of each specimen. These samples were homogenized, diluted in 50 mL of DYP medium (10 g of dextrose, 2.5 g of yeast extract and 5 g of peptone per litre of MilliQ water), and distributed in tubes. After 3 days of incubation at 25°C, each culture was serially diluted and 100 µL of each dilution was spread in DYP agar plates (DYP medium plus agar 16 g L⁻¹). The isolated microorganisms were tested for antibacterial activity as described below, and the strains that showed positive results were incubated in 350 mL of DYP medium. After growth, the medium was collected and filtered with a 0.22 µm membrane and the retained microorganisms were used for DNA extraction and molecular fingerprinting by amplification of target sequences. Amplification of 18S and 16S rRNA genes was performed using the primers ITS1 and ITS4 (White *et al.*, 1990), 27FB and 1492RAB (Turque *et al.*, 2008), and the products sequenced by capillary electrophoresis in a Megabace 1000 platform (GE Healthcare). Consensus sequences were then manually edited and analysed by BLAST searches at NCBI (National Center for Biotechnology Information). All sequences were deposited in GenBank.

Extraction of exudates from microorganism cultures

The filtered culture medium (350 mL) of each strain was passed through Sep-Pak C18 cartridges (Waters) and the analytes were eluted consecutively with ethanol 50% (ET50), ethanol 100% (ET100), hexane, dichloromethane and acetone. Each sample was evaporated in reduced pressure at 60°C, and the ethanol 50 and 100% fractions were lyophilized after the ethanol evaporation and stored at -20°C. The substances not retained by the Sep-Pak columns were passed through 10 and 1 kDa membranes. Smaller compounds were recovered from the filtrate by anion exchange chromatography with Amberlite IR-400 resin (Sigma). Each fraction was dried, weighed and stored at -20°C.

Antibacterial activity assay

The microorganisms used were *Staphylococcus aureus* (AS), *Salmonella enteritidis* (SE), *Pseudomonas aeruginosa* (PA), *Proteus vulgaris* (PV), *Klebsiella pneumoniae* (KP) and *Micrococcus luteus* (ML), which were incubated at 37°C for 24 h in Brain-Heart infusion (BHI) prior to the assays. The fractions obtained from the liquid culture were dissolved to 10 mg mL⁻¹ in 50% BHI for testing and the active extracts were dissolved to 2 mg mL⁻¹ for re-testing. The hydrophobic extracts were diluted in DMSO (dimethyl sulphoxide, 0.2 mg µL⁻¹) to permit the dilution in aqueous medium. In the assays, 50 µL of each culture (1 × 10⁸ CFU) were distributed in tubes with 400 µL of 50% BHI and 50 µL of the sample. For each treatment, controls without sample and a blank, without sample and microorganisms, were maintained. Aliquots (200 µL) of each test tube were read in 96-well plates, immediately prior to the test (*T*₀) as well as after 24 h of incubation (*T*₂₄). The optical density of the cultures was registered at 600 nm in a SpectraMax microplate reader (Molecular Devices), and the effect in the bacterial concentration was determined by comparing the optical density in the *T*₀ to the value registered in the *T*₂₄, for each treatment. The results (N = 9) were plotted as percentage using the

readings from the controls as baseline (zero) and the effects on the growth as positive (promoter) or negative (inhibitory) bars.

HPLC purification

The HPLC was performed in a LC-10 system connected to UV detector SPD-10 (Shimadzu) and a Capcell C18 column (Shiheidō). The extract showing highest antimicrobial activity (MERETb.762 ET100: 100 µg) was diluted in 50 µL of 50% acetonitrile and eluted with a gradient of acetonitrile (B) in 0.1% trifluoroacetic acid (A). B gradient was 0% (0–1 min), 0–100% (1–5 min), 100% (5–40 min), 100–0% (40–45 min) and 0% (45–60 min). The substances were detected at 214 nm wavelength and the flow rate was 1 mL min⁻¹. The fractions were manually collected, evaporated and stored at -20 °C until the degranulation assay.

Degranulation assay

The amount of β -hexosaminidase released by the cells was measured as described previously (Yan *et al.*, 2006). The rat basophilic leukaemia cells (RBL-2H3) were cultivated in Eagle medium with 15% foetal bovine serum. The cells were differentiated toward neutrophil-like upon incubation with dibutyryl-cAMP (0.2 mM) for 48 h and cultured overnight in 24-well tissue culture plates (2×10^5 per well). Then, washed twice with PBS and incubated 5 min with or without the HPLC fractions FII and FIV (10 µg mL⁻¹) and CsA (10 µM). Control cultures were incubated without the presence of inhibitors. After incubation for 15 min with formyl-methionyl-leucyl phenylalanine (fMLF, 100 nM) the degranulation was terminated by placing the plates on ice. The amount of β -hexosaminidase released into the medium was determined by incubating 20 µL of supernatant or cell lysate with 10 µL of 1 mM p-nitrophenyl-N-acetyl-D-glucosamide (pNAG) in sodium citrate buffer (0.1 M; pH 4.5) at 37 °C for 1 h. At the end of the incubation, 200 µL of 0.1 M Na₂CO₃ and 0.1 M NaHCO₃ (pH 10) were added and the absorbance was determined in a microplate reader at 405 nm. Total β -hexosaminidase was determined by lysing the RBL-2H3 cells in 0.1% Triton X-100. Data were collected from three independent experiments and presented as the percentage of the β -hexosaminidase released in relation to the total.

Mass spectrometry

Mass spectra were acquired on a LCMS-IT-TOF mass spectrometer (Shimadzu), equipped with a standard electrospray probe. The electrospray probe was adjusted to ~200 µL min⁻¹. The CDL and the heat block temperatures were maintained at 200 °C and the needle voltage at 3.6 kV, applying a drying gas flow (nitrogen) of 3 L min⁻¹ and a nebulizer gas flow (nitrogen) of 1.5 L min⁻¹. The mass spectrometer was calibrated with sodium trifluoroacetate and its typical cone-voltage induced fragments. About 50 pmol of each sample was injected into electrospray transport solvent. The ESI mass spectra were obtained in the continuous acquisition mode, scanning from m/z 50 to 1000 with a scan time of 7 s.

RESULTS

Fourteen bacterial and six fungal strains were isolated from the freshwater sponge samples. Two bacterial strains (MERETb.761, GenBank accession no KF305316; and MERETb.762, no KF305317) and one fungus (MERETf.010, no KF305318) showed natural antimicrobial activity. Both bacterial strains inhibited the fungus *Aspergillus* sp., that was grown in the same culture plate, producing an inhibition zone where the bacteria grew over the hypha (Figure 2). The fungus MERETf.010 was the only microorganism isolated from the number 10 sponge sample. An average of six to eight colonies of both bacteria and fungi were observed in each one of the other plates in these initial cultures, indicating that MERETf.010 strain inhibited all other microorganisms present in this sample. These three strains were then selected for further testing and cultured until the liquid medium acquired a dark brownish colour, which took about 45 days. In this stage, the cultures were then extracted and assayed for antibacterial activity as described in the experimental section.

The results showed that the ET050 and ET100 fractions from the fungus MERETf.010 and the bacteria MERETb.762 inhibited specifically the growth of *S. aureus* (10 mg mL⁻¹, Figure 3). Both ET100 fractions completely abolished bacterial growth. However, when the same fractions were tested in lower concentrations (2 mg mL⁻¹), only the bacterial ET100

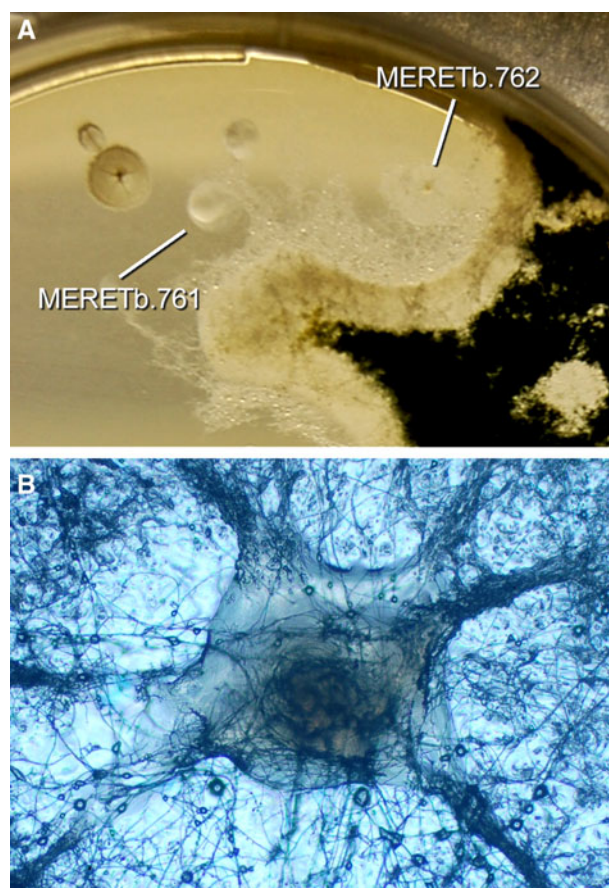


Fig. 2. (A) Inhibitory effect of the bacterial strains MERETb.761 and 762 against the fungus *Aspergillus* sp., both isolated from the same sponge sample (no. 7). (B) Detail of the bacteria MERETb.762 (opaque mass in the central area) growing over the fungal hyphae.

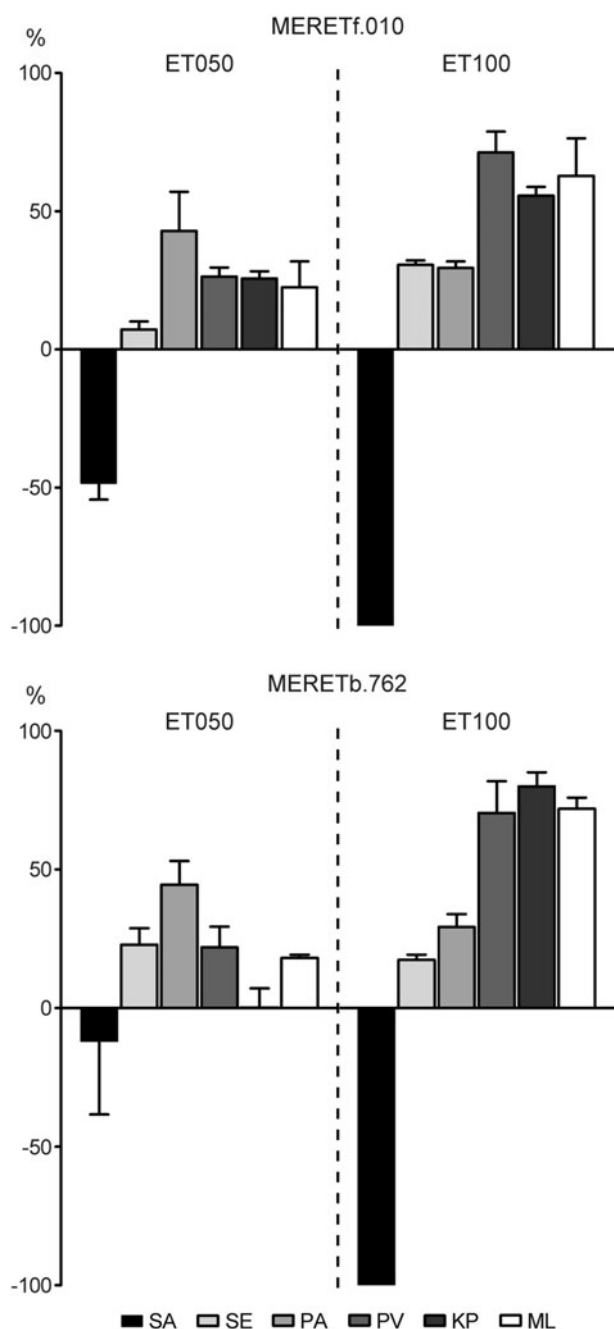


Fig. 3. Effects of the ethanol extracts ET050 and ET100 from the fungi MERETf.010 and the bacteria MERETb.762 on liquid cultures of *S. aureus* (SA), *S. enteritidis* (SE), *P. aeruginosa* (PA), *P. vulgaris* (PV), *K. pneumoniae* (KP) and *M. luteus* (ML). The results were plotted using the readings from the controls as baseline (zero) and the effects on the growth as positive (promoter) or negative (inhibitory) bars.

maintained the full inhibitory effect, while that from the fungi was slightly reduced ($97.2 \pm 2.8\%$).

Considering the specificity and the stronger antibiosis of ET100 from MERETb.762 against *S. aureus*, this fraction was then selected and analysed by HPLC. The HPLC profile of ET100 showed two main peaks, FII and FIV (Figure 4A), which were collected and tested in the RBL-2H3 degranulation assay. The results show that the fraction FII inhibited the β -hexosaminidase release down to $23.1 \pm 1.3\%$ and the FIV to $19.9 \pm 2.2\%$, similar to the CsA effect, with $22.5 \pm 0.9\%$ (Figure 4B). When these fractions were analysed by

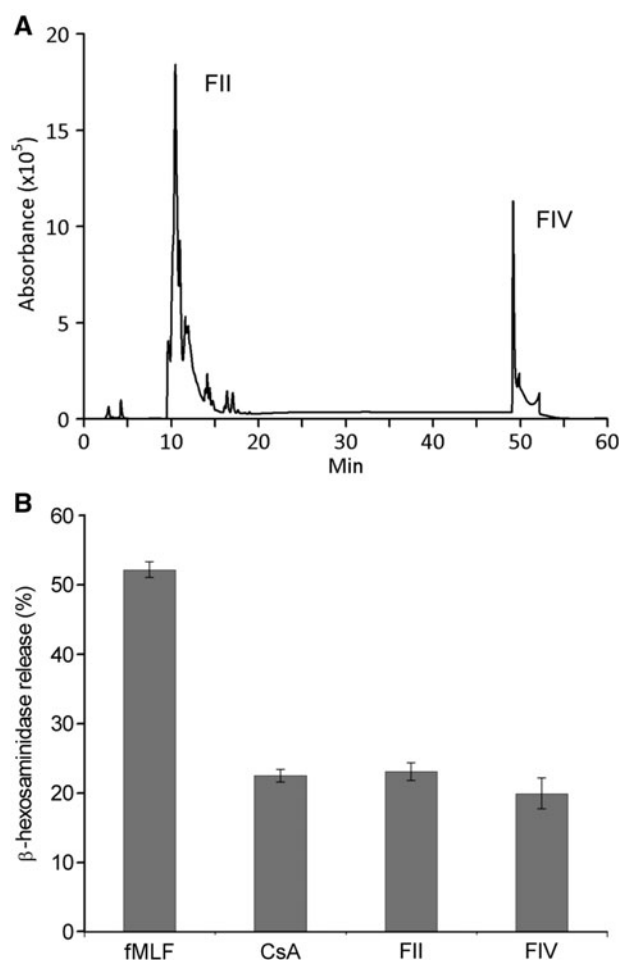


Fig. 4. (A) HPLC profile of the ethanol extract of the bacteria MERETb.762 culture medium, with the peaks FII and FIV. (B) Release of β -hexosaminidase by RBL-2H3 cells stimulated by fMLF and inhibitory activity of Cyclosporine A (CsA) and fractions FII and FIV on fMLF-stimulated cells. The results are presented as percentage of the total β -hexosaminidase in the cultures.

mass spectrometry, peaks of $168.0170 \text{ m z}^{-1}$ for FII and $215.5131 \text{ m z}^{-1}$ for FIV were observed, predicting the formulas $\text{C}_{12}\text{H}_6\text{N}_4\text{O}_8$ and $\text{C}_{25}\text{H}_4\text{N}_2\text{O}_6$, respectively, both corresponding to nitroaromatic compounds. The predicted formula for fraction FII corresponds to tetranitrophenyl, which is described as mutagenic in bacteria and carcinogenic in animal cells (Hirayama *et al.*, 1990; Purohit & Basu, 2000). There is no reference in the scientific literature for fraction FIV predicted formula. The FII and FIV were submitted to nuclear magnetic resonance analysis in the Analytical Center of Chemistry Institute (São Paulo University). However, the spectra were inconclusive due to the purity of compounds.

DISCUSSION

Brazil has a quite representative fauna of freshwater Porifera, with 52 of the 200 species described worldwide (Custódio & Hajdu, 2011). In several areas, these animals are an important fraction of the invertebrate biomass, known as *cauxi* by the local populations, and recognized as the cause of dermatitis

and other health problems (Volkmer-Ribeiro *et al.*, 2006; Cruz *et al.*, 2013). Nevertheless, the available literature is mostly limited to systematic or ecological studies, with a single article dealing with biochemical aspects (Barros *et al.*, 2013). This is the first report on bioactive substances obtained from freshwater sponges of the Amazon region, and this result can raise the awareness for these organisms in the region.

The inhibition induced by the substances isolated from the bacteria MERETb.762 can be acting through different pathways to exert the immunosuppressive effect. Cyclosporine A works by binding to cytosolic proteins, cyclophilins, and avoiding signal transduction from membrane receptors (Narenjkar *et al.*, 2006). However, CsA not only interfere with intracellular processes but also with the extracellular release of lytic components through degranulation induced by bacterial molecules (Passante & Frankish, 2009). The fMLF bacterial peptide activates the G protein and the downstream signalling molecules, and stimulates the RBL-2H3 degranulation (Yan *et al.*, 2006). This can be inhibited by the deactivation of the Ca^{2+} /calmodulin mediated by CsA. In addition, CsA also can suppress c-jun NH_2 -terminal kinase (JNK) and p38 mitogen-activated protein kinases (Funaba *et al.*, 2003).

A phylogenetic analysis based on the 16S rRNA gene of MERETb.762 (1404 nts) revealed similarities scores of 99% with several *Bacillus* strains, a common genus found in different marine sponges (Taylor *et al.*, 2007). The inhibition of degranulation evoked by fractions FII and FIV represent the first report of this activity for a sponge-associated bacterium. When the physiology of symbiotic organisms is analysed, the production of immunosuppressants is commonly mentioned as a possible strategy to avoid expulsion from their hosts (Corsaro *et al.*, 1999). The production of such compounds by MERETb.762 suggests a possible mechanism by which at least some sponge-associated microorganisms can maintain this relation.

The effects of several antimicrobial agents on the immune system, both at cell mediated and humoral levels, was recognized a long time ago (Finch, 1980; Reato *et al.*, 2004). However, this characteristic is not tested in screenings that usually focus only on antibiosis. Since sponges and their associated microbiota are known to produce numerous substances with such activity, immunosuppressive assays on newly isolated compounds could reveal new and interesting molecules.

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