Antimicrobial Activity of Heterotrophic Bacterial Communities from the Marine Sponge *Erylus discophorus* (Astrophorida, Geodiidae)

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Abstract

Heterotrophic bacteria associated with two specimens of the marine sponge *Erylus discophorus* were screened for their capacity to produce bioactive compounds against a panel of human pathogens (*Staphylococcus aureus* wild type and methicillin-resistant *S. aureus* (MRSA), *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Candida albicans* and *Aspergillus fumigatus*), fish pathogen (*Alivibrio fischeri*) and environmentally relevant bacteria (*Vibrio harveyi*). The sponges were collected in Berlengas Islands, Portugal. Of the 212 isolated heterotrophic bacteria belonging to Alpha- and Gammaproteobacteria, *Actinobacteria* and *Firmicutes*, 31% produced antimicrobial metabolites. Bioactivity was found mainly against *B. subtilis* and some bioactivity against *S. aureus* MRSA, *V. harveyi* and *A. fisheri*. No antifungal activity was detected. The three most bioactive genera were *Pseudovibrio* (47.0%), *Vibrio* (22.7%) and *Bacillus* (7.6%). Other less bioactive genera were *Labrenzia*, *Acinetobacter*, *Microbulbifer*, *Pseudomonas*, *Gordonia*, *Microbacterium*, *Micrococcus* and *Mycobacterium*, *Paenibacillus* and *Staphylococcus*. The search of polyketide I synthases (PKS-I) and nonribosomal peptide synthetases (NRPPs) genes in 59 of the bioactive bacteria suggested the presence of PKS-I in 12 strains, NRPS in 3 strains and both genes in 3 strains. Our results show the potential of the bacterial community associated with *Erylus discophorus* sponges as producers of bioactive compounds.

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Introduction

Due to their physico-chemical properties, which rarely exceed the biological tolerance limits, the oceans provide a safe environment for most living organisms [1]. However, the multiple beings that live in the marine environment had to develop survival strategies against other organisms with whom they have to compete for space and food. Sharing a common environment over a long evolutionary period also allowed the establishment of well-balanced associations between many of these organisms. A good example of these associations are the sponges that host a significant microbiome which can reach up to 40–60% of the total sponge biomass and densities of 10⁶ to 10¹⁰ bacteria per gram of sponge wet weight. These values can exceed seawater concentrations by two to three orders of magnitude [2–5].

Natural bioactive compounds have been used since the beginning of traditional medicine [6]. They are present in all kinds of life forms and are produced by the secondary metabolism of organisms. Secondary metabolites include terpenoids, alkaloids, polyketides, peptides, shikimic acid derivatives, sugars, steroids and a large mixture of biogenesis metabolites [7]. Sponge symbionts are fundamental in host defence against predators due to the production of biologically active secondary metabolites. These natural products can show antibacterial, antifungal, antiviral, antiprotozoal, anthelmintic, anti-inflammatory, antitumor, immunosuppressive, neurosuppressive properties and can also possess activities for the treatment of cardiac, respiratory and gastrointestinal diseases [8]. Sponges are the “gold mine” organisms for natural product isolation (over 30% of the products) in the marine environment [9]. The search for new drugs, especially antibiotics,
is important due to the increase of bacterial resistance to existing antibiotics.

The true origin of many of these bioactive molecules is uncertain. The production of secondary metabolites could be due to the cooperation between sponges and symbionts, only to the symbionts or to the sponges [10;11]. A microbial origin got support from the occurrence of structurally similar substances in unrelated sponges [see Laport et al. [8]]. The fact that these substances may be produced by microbes could allow their sustainable and unlimited production in vitro. This is hardly the case with sponges. As the sponge bioactivity may in fact be due to their microbiome, these organisms became the subject of many works.

Secondary metabolites possess complex structures and involve unusual biochemistry. Two families of enzymes, the polyketide synthases (PKS) and the nonribosomal peptide synthetases (NRPS), are of particular importance in the production of various secondary metabolites many of which are important drugs [12]. Both PKSs and NRPSs can be conceptualized as enzymatic “assembly lines” composed of functional modules [13].

The discovery of new biosynthetic pathways encoding genes of secondary metabolites opens the possibility of heterologous production and the genetic manipulation of the gene cluster to obtain new natural products [14]. Metagenomic analysis of the prevalence and presence of PKS and NRPS genes are being studied to improve the search of new bioactive compounds in sponges (e.g. Schirmer et al. [15]).

Sponges belonging to the genus *Erylus* (Astrophorida, Geodidaceae), are well known as producers of saponins and other oligoglycosides [16–27]. These natural products have been reported to exhibit a wide spectrum of biological activities which include selective thrombin receptor antagonist activity and functional activity in a platelet aggregation assay [19], immunopressive activity [20], inhibition of neuraminidase from the bacterium *Clostridium perfringens* [21] and antitumor and antifungal properties [16,23,24]. Alcoholic extracts of *Elylus deficiens* Topsent, 1927 from the Mediterranean sea showed antibacterial activity against the marine bacteria *Alteromonas luteo-violacea* and 8 terrestrial bacteria (*Staphylococcus epidermidis, Sarcina lutea, Bacillus subtilis, Micrococcus luteus, Serratia marcescens, Enterobacter sp.*, *Proteus morganii* and *Proteus mirabilis*) [29]. Antimicrobial activity against *Bacillus subtilis, Escherichia coli* and *Candida albicans* was observed in the extracts of *Elylus lendenfeldi* Sollas, 1888 collected in the Red Sea [22]. In addition, multiple ecological functions have also been attributed to these molecules. Furthermore, it was shown that the sponge *Elylus formosa* Sollas, 1886 contained sufficiently high concentrations of erylosides to protect itself against predatory fishes, bacterial settlement, and fouling [30–31]. These natural compounds have been isolated from different species of *Elylus* as well as from different geographic locations [23–27]. Another species, *E. discophorus* Schmidt, 1862, was reported to have haloperoxidase with isoo- and bromoperoxidases activities [32].

In this study we aimed to assess the bioactive potential of bacteria isolated from the marine sponge *E. discophorus* collected in Berlengas Islands against a panel of pathogenic and environmental microorganisms. Antimicrobial bioactivity was detected in 31% of the 212 isolated heterotrophic bacteria from two specimens of *E. discophorus* and the presence of PKS-I and NRPSs genes was detected in several isolates showing the biotechnological potential of these bacteria.
generated were employed to seed all subsequent micro-fermentations carried out in both Janus and Duetz plates.

2.1 Janus plates assays. This double-faced plated assay optimized by Fundación MEDINA [37] is based in exclusively designed plates by Nunc with two sides, which allow culture growth on the opposite layers in solid media. In order to maximize the number of potential active secondary metabolites produced, 5 different media were chosen to carry out the miniaturized fermentations: Marine Broth (2216 Difco), Medium F (0.015 g K$_2$HPO$_4$; 0.2 g CaCl$_2$; 0.75 g KCl; 23.4 g NaCl; 7 g MgSO$_4$; 1 g Mannitol; 1 g Yeast extract; 1 g Peptone; 16 g Agar; 1 ml Hutter’s basal salts [33] and 999 ml deH$_2$O), R2A (218263 - Difco) and saline (supplemented with 3% of SeaSalts of Sigma) R2A and Starch agar (Difco).

On the top layer of the Janus plates the test bacteria were inoculated employing a replication procedure previously described [36] and incubated for 3 days at 28°C and 70% of humidity.

Subsequently, the inoculated media with the target microorganisms were added on the opposite side thus forming the assay layer. All target organisms were pre-inoculated and grown at 28°C, 220 r.p.m. for 12 h and 70% of humidity. Pre-inoculum and inoculum medium used to grow A. baumannii and B. subtilis was Luria Broth (LB) (Miller’s, Invitrogen). For C. albicans the pre-inoculum medium was Sabouraud Dextrose Broth (SDB) and the inoculum medium was YNBD [37]. The pre-inoculum and inoculum medium prepared for the overnight growth of S. aureus MRSA was Brain Heart Infusion (BHI). Inocula absorbance for all target microorganisms was adjusted to a final 600 nm optical density (OD) of 0.4 before being placed in the Janus plates. Non-inoculated medium was used as negative control.

Tests of growth interference of the target microorganisms with saline media were performed in advance to rule out possible interferences.

2.2 Microfermentations in 96-deep well plates. MPs were used to carry out microfermentations in 96-deep well plates (here designated by Duetz system assay) following the approach described by Duetz et al. [36]. For this assay, besides the 5 media already specified for the Janus plates, half saline concentration of the media were also tested. The inoculated Duetz plates were incubated (1 mL) for 5 days at 28°C, 220 r.p.m. and 70% of humidity. Bacterial broth were then subjected to an organic extraction with 800 µl acetone and 40 µl DMSO per well. The plates were incubated for 1 h at 220 r.p.m. and then transferred to a vacuum centrifuge (GeneVac HT-24) in order to reduce the final volume to 400 µl (2 Whole Broth Equivalent). The supernatants of the extracts solutions were transferred to 96-deep well plates. The organic extracts were then assayed against the Gram-negative Pseudomonas aeruginosa PAO1, Acinetobacter baumannii, Vibrio harveyi (CECT 525) and Aliivibrio fisheire (CECT 524); and the Gram-positive Bacillus subtilis (ATCC6633), Staphylococcus aureus wild type Smith strain and Staphylococcus aureus MRSA. Antibacterial assays were also prepared against the yeast Candida albicans and Aspergillus fumigatus (ATCC46645) and (Δzid$_3$[138]). Unless specified negative controls were always uncultivated control medium.

For the screening assays against Acinetobacter baumannii, S. aureus (Smith), Pseudomas aeruginosa PAO1 and Candida albicans, overnight cultures grown in liquid Luria Broth (Miller’s media) at 37°C and 220 r.p.m. were measured at an absorbance of 612 nm for A. baumannii and S. aureus (Smith) and 600 nm for P. aeruginosa. The inocula in LB media were adjusted to an OD of 5×10$^{-3}$ for A. baumannii and to a cell concentration of 2.5×10$^5$ CFU/ml for S. aureus (Smith) and 5×10$^5$ CFU/ml for P. aeruginosa. A suspension of C. albicans in medium RPMI (40 ml 1 M HEPES; 36 ml 50% glucose; 6.7 g Yeast Nitrogen Broth without amino acids; RPMI a bottle; adjust pH to 7.1 and 0.22 µm sterilized) was adjusted to 0.250 at 660 nm. This suspension was then diluted 1/10.

For the liquid screens, Nunc plates (LTC-330) were filled as follows: 10 µl of each extract plus 90 µl of inoculum in 80 wells; 10 µl of a series of antibiotic concentrations in the range of the minimum inhibitory concentration plus 90 µl of inoculum (positive and negative controls) in 8 wells; 100 µl (90 µl of LB media +10 µl DMSO 20%) as blank control in 4 wells; and 100 µl of the inoculum for the assessment of the total microorganism growth.

The antibiotics used against A. baumannii were, as positive controls, 0.3125, 0.625, 12.5 and 25 µg.mL$^{-1}$ rifampicin and, as negative controls, 7.8125, 15.625, 31.25 and 62.5 µg.mL$^{-1}$ amphotericin B. In the case of P. aeruginosa the positive controls were 1.9625, 3.925, 7.85 and 15.7 µg.mL$^{-1}$ ciprofloxacin and the negative controls were 7.8125, 15.625, 31.25 and 62.5 µg.mL$^{-1}$ amphotericin B. In the assays performed with S. aureus (Smith) the positive controls used were serially diluted between 0.5×10$^{-7}$ to 5 mg.mL$^{-1}$ penicillin G and with C. albicans the antibiotics used were as positive controls 7.8125, 15.625, 31.25 and 62.5 µg.mL$^{-1}$ amphotericin B and as negative controls 0.039, 0.078, 0.156 and 0.312 mg.mL$^{-1}$ penicillin G.

The plates were incubated at 37°C for 20 h under humid conditions. The absorbances were measured at 612 nm in a Tecan ULTRA EVOLUCTION before and after incubation. To confirm the results, 30 µl of a 0.02% resazurin stock solution was added to each well (100 µl) and incubated for 2 h. Changes in colour from blue (growth inhibition corresponding to detection of bioactivity) to pink (no growth inhibition corresponding to no detection of bioactivity) and fluorescence readings radiated from the resazurin were measured in a Perkin Elmer VICTOR2 multi-function fluorometer. All ODs and fluorescence measurements were analysed using the Genedata Screener software.

For the screening assays against Aspergillus fumigatus ATCC46645 and Δzid$_3$[138], cultures of both A. fumigatus were prepared in medium RPMI with 0.002% resazurin from a stock powder suspension in Tween saline buffer to a final concentration of 2.5×10$^3$ conidia/ml as described by Monteiro et al. [39]. The assays were carried out as previously described, using as positive controls 0.5, 1.0, 2.0 and 4.0 µg.mL$^{-1}$ amphotericin B. The plates were incubated for 30 h at 37°C and, then, the fluorescence was recorded in a Perkin Elmer VICTOR2™ Multi-function fluorometer.

For the screening assays against Staphylococcus aureus MRSA, an overnight culture of S. aureus MRSA in 10 ml of liquid Brain Heart Infusion (BHI) medium was incubated at 37°C and 220 r.p.m. The OD of the culture measured at 660 nm was adjusted to 0.2 and used to inoculate BHI agar medium (3 ml of the adjusted inoculum per 100 ml of medium) which was then distributed (30 ml) in OmnyTray plates. Disposable 96 pin trays were used to generate 96 wells in each of the BHI agar plates in which, subsequently, 10 µl of each extract in each well was dispensed. Alternatively extracts were distributed in BHI agar plates without wells. Ten µl of 0.5 mg.mL$^{-1}$ kanamycin was used as positive control. Plates were then incubated at 37°C for 20 h and zones of inhibition (ZOI) were measured. Any extract producing a visibly discernible ZOI, regardless of zone quality, was considered to be positive.
For the screening assays against *Bacillus subtilis* (ATCC 6633), a culture of *B. subtilis* was prepared using 1 ml of spore suspension/1 L medium (23 g/L of Nutrient Agar and 2 g/L of yeast extract) that had been previously sonicated for 3 min. The assay was performed in a similar way to the one used against *S. aureus* MRSA and the positive control was 150 μg/ml tunicamycin.

Screening assays against *Vibrio harveyi* CECT 525 and *Aliivibrio fischeri* CECT 524 were carried out in a cell density optimized agar assay. Ten ml of Luminous Medium [40] were inoculated with a loop of the pure *V. harveyi* and *A. fischeri*, incubated at 25°C, 220 r.p.m. for 12 h and the optical density adjusted to 0.3 for *V. harveyi* and 0.4 for *A. fischeri* at 600 nm and, subsequently, both diluted by 1/10. The assay was performed in a similar way to the one used against *S. aureus* MRSA and the positive control consisted on 0.256 mg/ml chloramphenicol. The cultures were incubated at 25°C for 24 h. Inhibition was detected with the presence of non-phosphorescent halos in a dark-room.

3. Search of PKS and NRPS genes

The molecular analysis of the genes PKS-I and NRPS involved in the production of secondary metabolites was investigated in the 59 of the bioactive bacteria. Genomic DNA was extracted with an E.Z.N.A. bacterial KIT from OMEGA. Specific degenerated primers MDPOQQR1 and HDCTGTR [41] and DKT and MTR [42] were used for PCR amplification of PKS-I and NRPS genes, respectively. A total of 25 μl of PCR mixture (1 × PCR buffer with 1.7 mM MgCl2; 0.8 unit of Go Taq DNA Polymerase; 0.2 mM of each dNTPs; 0.1 μM of each primer and 5 μl DNA template) was used. The PCR program for the genes PKS-I and NRPS was the same and consisted of an initial denaturing step of 5 min at 95°C; 11 cycles of 1 min at 95°C; 30 s at 60°C and 1 min at 72°C, with the annealing temperature reduced by 2°C per cycle, followed by 30 cycles of 95°C for 1 min, 40°C for 30 s and 72°C for 1 min with a final extension of 10 min at 72°C. The PCR programs were performed in an MyCycler™ Thermo Cycler (Bio-Rad) and the PCR products were visualized in VWR GenoPlex after electrophoresis in a 1.2% agarose gel in 1X TAE buffer.

Results and Discussion

The oceans, an almost endless source of microbial diversity, are the habitat of organisms such as sponges that harbour a large microbial diversity with important biosynthetic potential due to their secondary metabolites profiles [43]. The analysis of sponge symbionts in pure cultures is an advantage for the performance of bioactive screening assays [44] and is the most direct method for the large-scale production of bioactive compounds [45]. The two specimens of *Erylus discophorus* collected in Berlangas (Berg01 and Berg02) allowed the isolation of a large number of heterotrophic bacteria (212 isolates) of which 31% (66 isolates) showed bioactivity. Of the screened bacteria for bioactivity and based on the 16S rDNA gene analysis, 57% (n = 120) were Alphaproteobacteria, 21% (n = 45) Gammaproteobacteria, 16% (n = 34) Actinobacteria and 6% (n = 13) Firmicutes. Bioactivity was observed against one or more of the target microorganisms tested. The majority was active against *Bacillus subtilis* (87.9%) and at a lower percentage against *Staphylococcus aureus* MRSA (10.6%), *Aliivibrio fischeri* (9.1%) and *Vibrio harveyi* (6.1%). Bacteria with bioactivity against both *B. subtilis* and *S. aureus* MRSA represented 9.1% and against both *A. fischeri* and *V. harveyi* accounted for 4.6%. No bioactivity was observed against *P. aeruginosa*, *A. baumannii*, *S. aureus* wild type, *C. albicans* and *A. fumigatus*.

The taxonomic affiliation of all bioactive isolates is provided in Table 1.

Table 2 correlates the taxonomic position of the isolates genera and their relative bioactivity percentage. It also specifies the number and relative percentage of bioactive isolates obtained with the *Janus* and Duetz systems. Thirty two isolates of Alphaproteobacteria (48.5%) belonging to the genera *Pseudovibrio* and *Labrenzia* were bioactive against *B. subtilis*, *S. aureus* MRSA and *A. fischeri*. Eighteen isolates of Gammaproteobacteria (27.3%) belonging to the genera *Vibrio*, *Achromobacter*, Microbulbifer and *Pseudomonas* were active against *B. subtilis*, *V. harveyi* and *A. fischeri*. Eight isolates of Actinobacteria (12.1%) belonging to the genera *Gordonia*, *Microbacterium*, *Micromussa* and *Mycobacterium* were active against *B. subtilis* and *S. aureus* MRSA. Eight isolates of Firmicutes (12.1%) belonging to the genera *Bacillus*, *Paenibacillus*, *Sporosarcina* and *Staphylococcus* were active against *B. subtilis*, *V. harveyi* and *A. fischeri*. *Pseudovibrio* (47.0%), *Vibrio* (22.7%) and *Bacillus* (7.6%) are the three most bioactive genera of all the bioactive isolates. No activity was observed in isolates affiliated to *Ruegeria*, *Rhodobacter*, *Erythrobacter*, *Marateella*, *Natella*, *Photobacterium*, *Thalassomonas*, *Rhodosaccus* and *Dietzia*.

The group with the higher number of isolates demonstrating bioactivity was the Alphaproteobacteria followed by the Gammaproteobacteria, *Actinobacteria* and *Firmicutes*. However, if the analysis is made based on the number of bioactive isolates relative to the total number of bacteria in each group, *Firmicutes* are the most bioactive (61.5%) followed by Gammaproteobacteria (40%), Alphaproteobacteria (26.7%) and *Actinobacteria* (25.5%). Bioactivity results obtained with marine bacteria and sponge associated bacteria are somehow different. Regarding marine bacteria in general, most of the new marine bacterial compounds from 1997 to 2008 were originated from *Actinobacteria* (40%), *Cyanobacteria* (33%), *Proteobacteria* (12%) and *Firmicutes* and *Bacteroidetes* (5% each) [46]. The distribution of bioactive compounds produced by sponge associated bacteria is *Actinobacteria* (46.66%), *Proteobacteria* (23.33%), *Firmicutes* (11.66%), *Cyanobacteria* (8.33%), *Verrucomicrobia* (5%) and others (5%) [47]. The high number of bioactive *Actinobacteria* may be biased due to their extensive study in the production of antibiotic compounds since 50% of known microbial antibiotics are derived from these bacteria [48].

Several of the obtained bioactive genera are well known producers of metabolites with antimicrobial properties but others are less known. Furthermore, many of the examples referred to below are from sponge associated bacterial isolates.

A suite of antimicrobial compounds with spectra of different antimicrobial activity was observed in *Pseudovibrio* [11:49–52]. A sponge associated *Alphaproteobacterium* related to *Pseudovibrio denitrificans*, displayed a weak and unstable antimicrobial activity, which was easily lost during cultivation [53]. However, this bioactive bacterium was present in the sponges in high numbers. High antimicrobial activity was also observed in isolates from soft corals affiliated to the alaphaproteobacterium *Labrenzia* [51].

Marine vibrios have been reported as a rich source of novel biologically active metabolites [10;51,54–57]. Bioactivity produced by *Vibrio* sp. and sponge extracts was observed against *Bacillus* [58]. A total of 93 secondary metabolites were isolated from *Vibrioaceae* [59]. These are surface-associated bacteria known to produce a broad range of antibacterial compounds which may have a relevant ecological role favouring their abundance in microbial communities [59].

Bioactive metabolites produced by marine *Pseudomonas* species have been reported [10;54;55;60–62]. Marine *Pseudomonas* spp. as potential source for medically relevant bioactive substances were revised by Isnansetyo and Kamei [63].
| Isolate | Closest strain; Accession no. | Similarity | Genera | Phylum/Class |
|---------|-----------------------------|------------|--------|--------------|
| Berg02_22.2 | Gordonia sp. DE08200; AY927227 | 100.0% | Gordonia | Actinobacteria |
| Berg02_29 | Gordonia sp. CN1786 PLO4; DQ448772 | 98.6% | Gordonia | Actinobacteria |
| Berg02_78 | Gordonia terrae; 3269aBRRJ; FJ200386 | 99.6% | Gordonia | Actinobacteria |
| Berg02_79 | Microbacterium sp. M63-2; EF061897 | 98.8% | Microbacterium | Actinobacteria |
| Berg02_79a | Microbacterium sp. M63-2; EF061897 | 99.7% | Micrococcus | Actinobacteria |
| Berg02_11 | Micrococcus sp. LIYS; EU379020 | 99.7% | Micrococcus | Actinobacteria |
| Berg02_26 | Micrococcus luteus; KCL-1; DQ538135 | 98.7% | Micrococcus | Actinobacteria |
| Berg01_46 | Mycobacterium sp. JL838; DQ985057 | 96.9% | Mycobacterium | Actinobacteria |
| Berg02_114.2 | alpha proteobacterium CRA 405; AY562568 | 96.4% | Labrenzia | Alphaproteobacteria |
| Berg01_7 | Pseudovibrio ascidiaceicola | 100.0% | Pseudovibrio | Alphaproteobacteria |
| Berg01_9 | Pseudovibrio ascidiaceicola (T); F423(= NBRC 100514); AB175663 | 99.8% | Pseudovibrio | Alphaproteobacteria |
| Berg01_16 | sponge bacterium Isolate3; AY948383 | 98.3% | Pseudovibrio | Alphaproteobacteria |
| Berg01_33 | alpha proteobacterium CRA 3GB; AY562562 | 96.2% | Pseudovibrio | Alphaproteobacteria |
| Berg01_50 | alpha proteobacterium CRA 3GB; AY562562 | 99.6% | Pseudovibrio | Alphaproteobacteria |
| Berg01_68 | Pseudovibrio sp. B411; FN295808 | 98.1% | Pseudovibrio | Alphaproteobacteria |
| Berg01_77 | Pseudovibrio sp. PV4; EU768841 | 98.9% | Pseudovibrio | Alphaproteobacteria |
| Berg02_8.1 | alpha proteobacterium CRA 3GB; AY562562 | 98.6% | Pseudovibrio | Alphaproteobacteria |
| Berg02_8.3 | sponge bacterium Isolate1; AY948382 | 93.7% | Pseudovibrio | Alphaproteobacteria |
| Berg02_9.1 | alpha proteobacterium CRA 3GB; AY562562 | 99.4% | Pseudovibrio | Alphaproteobacteria |
| Berg02_10.1 | alpha proteobacterium PV4; EU768841 | 99.2% | Pseudovibrio | Alphaproteobacteria |
| Berg02_10c | alpha proteobacterium CRA 3GB; AY562562 | 99.3% | Pseudovibrio | Alphaproteobacteria |
| Berg02_13.1 | Pseudovibrio sp. PV4; EU768841 | 99.2% | Pseudovibrio | Alphaproteobacteria |
| Berg02_17 | Pseudovibrio sp. PV4; EU768841 | 98.7% | Pseudovibrio | Alphaproteobacteria |
| Berg02_18 | sponge bacterium Isolate1; AY948382 | 96.0% | Pseudovibrio | Alphaproteobacteria |
| Berg02_36 | alpha proteobacterium CRA 3GB; AY562562 | 97.1% | Pseudovibrio | Alphaproteobacteria |
| Berg02_39.1 | sponge bacterium Isolate3; AY948383 | 98.3% | Pseudovibrio | Alphaproteobacteria |
| Berg02_39.3 | sponge bacterium Isolate1; AY948382 | 99.5% | Pseudovibrio | Alphaproteobacteria |
| Berg02_40 | sponge bacterium Isolate1; AY948382 | 98.4% | Pseudovibrio | Alphaproteobacteria |
| Berg02_41 | alpha proteobacterium CRA 3GB; AY562562 | 98.8% | Pseudovibrio | Alphaproteobacteria |
| Berg02_50 | alpha proteobacterium CRA 3GB; AY562562 | 96.4% | Pseudovibrio | Alphaproteobacteria |
| Berg02_50a | alpha proteobacterium CRA 57G; AY562561 | 96.6% | Pseudovibrio | Alphaproteobacteria |
| Berg02_54.1 | Pseudovibrio sp. PV4; EU768841 | 99.3% | Pseudovibrio | Alphaproteobacteria |
| Berg02_56.2 | alpha proteobacterium CRA 3GB; AY562562 | 98.6% | Pseudovibrio | Alphaproteobacteria |
| Berg02_57 | sponge bacterium Isolate3; AY948383 | 98.3% | Pseudovibrio | Alphaproteobacteria |
| Berg02_58 | Pseudovibrio sp. PV4; EU768841 | 98.8% | Pseudovibrio | Alphaproteobacteria |
| Berg02_61 | Pseudovibrio ascidiaceicola (T); F423(= NBRC 100514); AB175663 | 97.4% | Pseudovibrio | Alphaproteobacteria |
| Berg02_63 | sponge bacterium Isolate1; AY948382 | 99.4% | Pseudovibrio | Alphaproteobacteria |
| Berg02_65 | Pseudovibrio ascidiaceicola (T); F423(= NBRC 100514); AB175663 | 100.0% | Pseudovibrio | Alphaproteobacteria |
| Berg02_141 | sponge bacterium Isolate1; AY948382 | 99.6% | Pseudovibrio | Alphaproteobacteria |
| Berg02_188 | sponge bacterium Isolate3; AY948383 | 98.0% | Pseudovibrio | Alphaproteobacteria |
| Berg01_47 | Bacillus circulans | 99.0% | Bacillus | Firmicutes |
| Berg01_48 | Bacillus circulans (T); AYO43084 | 97.9% | Bacillus | Firmicutes |
| Berg01_114 | Bacillus sp. enrichment culture clone SYW22; FJ601652 | 99.9% | Bacillus | Firmicutes |
| Berg02_107 | Bacillus megaterium; MO31; AY553118 | 100.0% | Bacillus | Firmicutes |
| Berg02_161a | Bacillus sp. RS654(2010); GU968484 | 99.6% | Bacillus | Firmicutes |
| Berg01_119 | Paenibacillus sp. 1 GUV; EU496552 | 99.6% | Paenibacillus | Firmicutes |
| Berg02_117 | Sporosarcina luteola | 99.9% | Sporosarcina | Firmicutes |
The production of antibacterial compounds by a halotolerant *Acinetobacter* sp. from salt pans of Ribandar, Goa was observed [64]. *Acinetobacter* from the ascidian *Stomozoa murrayi* [65] also produced bioactive compounds. *Microbulbifer* produce anticancer antibiotics (pelagiomics) [66] and a broad variety of natural parabens [67]. Further evidence of production of antimicrobial activity by *Microbulbifer* was obtained by Penesyan et al. [68].

*Bacillus* spp. from terrestrial origin are well known sources of antimicrobial compounds [69]. Similarly antibiotics/bioactive compounds from marine *Bacillus* spp. have also been reported [10;53;55–57;70;71]. Genome sequencing studies of the genus *Bacillus* have revealed its potential as a source of antibiotic-like compounds [72].

Antimicrobial activity of the Gram negative strains *Paenibacillus* sp., *P. elgii*, *P. polymyxa* and *P. koreensis* has been observed [70;73–75]. Anand et al. [55] observed bioactivity in *Staphylococcus* bacteria isolated from four species of sponges.

*Micrococcus* strains possessing antimicrobial activity were described by Bultel-Poncè et al. [61], Hentschel et al. [76] and Lo Giudice et al. [77]. Antitumor and antimicrobial bioactivity was observed by *Microbacterium* species [57;78;79]. In a recent review the actinomycete genus *Gordonia* was described as being capable of degrading xenobiotics, environmental pollutants, or otherwise slowly biodegradable natural polymers as well as to transform or synthesize possibly useful compounds [80]. However, to our knowledge, no antimicrobial activity has been associated with this genus, nor with the *Mycobacterium* genus which is well known for its infectivity.

The most efficient liquid medium for the production of bioactive compounds against *B. subtilis*, *S. aureus* MRSA, *V. harveyi* and *A. fischeri* was MB. In saline R2A bioactivity was observed against both *S. aureus* MRSA and *V. harveyi*, and in saline Starch and MF against *A. fischeri*. In solid media assays, higher numbers of inhibitions were observed in saline Starch followed by MF and saline R2A. No inhibitions were found in MA medium when used as culture medium in the *Janus* system. The non-saline R2A medium was the only one where no bioactive compounds were produced in both systems.

The screening of the 212 bacteria was performed using two different plate systems. The Duetz system is a miniaturized fermentation system that allows carrying out a high number of microfermentations with a lot less effort than the effort required to carry out the fermentations in tubes/flasks. Bacteria are fermented in liquid media, crude extracts are obtained and assayed for bioactivity against target organisms. However, in the double-faced diffusion assay *Janus* system, after the initial growth of the sponge isolates, the medium, containing diffused secondary metabolites is put in contact with the target organism to assess inhibitory responses. The *Janus* system allowed the assessment of a higher number of bioactive positive hits (84%) when compared to the Duetz system (16%). However, the Duetz results are more reliable than the ones obtained with the *Janus* plates due to the overlap of inhibition halos in the latter. Moreover, due to bacterial swarming and gliding, results in the *Janus* system were obtained after 3 days of incubation, whereas longer incubation times were employed in the liquid microfermentations. Bacterial swarming and gliding also interfered with the visualization of results in other antimicrobial studies [37;53].

It is well known that microbial secondary metabolite production is highly-dependent on the fermentation conditions [81]. Growth media and growth conditions are variables that are known to have effects on the production of bioactive compounds and can be different depending on the strains [82]. As the production of secondary metabolites is mainly observed in late exponential/stationary phases, bacteria should produce more bioactive compounds after 5 days than 3. This hypothesis could be true for some bacteria and may explain some of the different bioactivity percentages obtained with the two methods. Furthermore, being
| Phylum/Class       | Closest Genus | Number of isolated strains | Number of bioactive strains | % of bioactive strains in the 212 bacteria | Number of bioactive strains in Janus system | % of bioactive strains in Janus system | Number of bioactive strains in Duetz system | % of bioactive strains in Duetz system | Number of bioactive strains in both Janus and Duetz system | % of bioactive strains in both Janus and Duetz system |
|-------------------|--------------|----------------------------|-----------------------------|-------------------------------------------|-------------------------------------------|----------------------------------------|------------------------------------------|----------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Actinobacteria    | Dietzia      | 9                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Gordonia     | 8                          | 3                           | 4.55                                      | 3                                         | 4.55                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Microbacterium | 3                         | 2                           | 3.03                                      | 2                                         | 3.03                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Micrococcus  | 6                          | 2                           | 3.03                                      | 2                                         | 3.03                                   | 1                                        | 1.52                                   | 1                                               | 1                                               |
|                   | Mycobacterium | 5                         | 1                           | 1.52                                      | 1                                         | 1.52                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Rhodococcus  | 3                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Total of Actinobacteria | 34                     | 8                           | 12.12                                     | 8                                         | 12.12                                  | 1                                        | 1.52                                   | 1                                               | 1                                               |
| Alphaproteobacteria | Pseudovibrio   | 61                         | 31                          | 46.97                                     | 25                                        | 37.88                                  | 9                                        | 13.64                                  | 3                                               | 3                                               |
|                   | Labrenza      | 9                          | 1                           | 1.52                                      | 1                                         | 1.52                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Ruegeria      | 44                         | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Rhodobacter   | 2                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Erythrobacter | 2                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Martelella    | 1                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Nautella      | 1                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Total of Alphaproteobacteria | 120                  | 32                          | 48.48                                     | 26                                        | 39.39                                  | 9                                        | 13.64                                  | 2                                               | 2                                               |
| Firmicutes        | Bacillus      | 10                         | 5                           | 7.58                                      | 2                                         | 3.03                                   | 3                                        | 4.55                                   | 0                                               | 0                                               |
|                   | Paenibacillus | 1                          | 1                           | 1.52                                      | 0                                         | 0.00                                   | 1                                        | 1.52                                   | 0                                               | 0                                               |
|                   | Sporosarcina  | 1                          | 1                           | 1.52                                      | 0                                         | 0.00                                   | 1                                        | 1.52                                   | 0                                               | 0                                               |
|                   | Staphylococcus | 1                         | 1                           | 1.52                                      | 1                                         | 1.52                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Total of Firmicutes | 13                     | 8                           | 12.12                                     | 3                                         | 4.55                                   | 5                                        | 7.58                                   | 0                                               | 0                                               |
| Gammaproteobacteria | Acinetobacter | 2                          | 1                           | 1.52                                      | 1                                         | 1.52                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Micebulbifer  | 7                          | 1                           | 1.52                                      | 0                                         | 0.00                                   | 1                                        | 1.52                                   | 0                                               | 0                                               |
|                   | Pseudomonas   | 6                          | 1                           | 1.52                                      | 1                                         | 1.52                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Photobacterium | 1                         | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Thalassomonas | 1                          | 0                           | 0.00                                      | 0                                         | 0.00                                   | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Vibrio        | 28                         | 15                          | 22.73                                     | 15                                        | 22.73                                  | 0                                        | 0.00                                   | 0                                               | 0                                               |
|                   | Total of Gammaproteobacteria | 45                     | 18                          | 27.27                                     | 17                                        | 25.76                                  | 1                                        | 1.52                                   | 0                                               | 0                                               |
|                   | Total         | 212                        | 66                          | 100.00                                    | 54                                        | 81.82                                  | 16                                       | 24.24                                  | 4                                               | 4                                               |

Table 2. Results of bioactivity by genera and screening method (Janus and Duetz systems) obtained against *Bacillus subtilis*, *Staphylococcus aureus* MRSA, *Aliivibrio fisheri* and *Vibrio harveyi*. doi:10.1371/journal.pone.0078992.t002
strains should amplify with other specific primers for the PKS-I and NRPS in 3 strains (Table 3). The non-amplification of PKS-I was observed in 12 strains, NRPS in 3 strains and both or two of the genes for secondary metabolites. The presence of these organisms, namely by the production of secondary metabolites like PKS-II gene [83].

The majority of the bioactive bacteria that amplified PKS-I and NRPS genes belongs to the genus Pseudovibrio (n = 10). These genes were already reported in Pseudovibrio strains isolated from a Irciniidae sponge [84;85]. Although a high number of the bioactive bacteria belongs to the genus Vibrio, only 3 bacteria amplified the PKS-I gene and none the PKS genes. A recent review on the family Vibrionaceae suggests that only NRPS or hybrid PKS-NRPS genes were amplified [59]. Furthermore, the genus Gordonia (n = 2), Bacillus (n = 2) and Pseudomonas (n = 1) amplified the genes NRPS and PKS in lower numbers. PKS and NRPS genes have been extensively studied in Actinobacteria of the genera Streptomyces, Mycobacterium, Corynebacterium, Micrococcus and Gordonia [86–91]. A wide range of bacterial groups were tested for the presence of the genes PKS and NRPS and they were found among other genera in Pseudomonas, Vibrio and Bacillus [93].

These results confirm the production of secondary active metabolites by some Erylus strains that are, thus, the most promising ones for future work. The complex bacterial communities in marine sponges play a considerable ecological role in several aspects of the biology of these organisms, namely by the production of secondary metabolites that could be bioactive.

Table 3. Presence of PKS-I and NRPS genes in the bioactive E. discophorus bacteria.

| Isolate   | Closest strain; accession no. | Genera           | Gene present     |
|-----------|-------------------------------|------------------|------------------|
| Berg02.22 | Gordonia sp. DEOB200; AY927227 | Gordonia         | PKS-I            |
| Berg02.78 | Gordonia terrae; 3269aBRR1; FJ200386 | Gordonia         | NRPS/PKS-I       |
| Berg01.7  | Pseudovibrio ascidicacecola   | Pseudovibrio     | PKS-I            |
| Berg01.9  | Pseudovibrio ascidicacecola (T); F423(= NBRC 100514); AB175663 | Pseudovibrio     | PKS-I            |
| Berg01.16 | sponge bacterium Isolate3; AY948383 | Pseudovibrio     | PKS-I            |
| Berg01.33 | alpha proteobacterium CRA 3GB; AY562562 | Pseudovibrio     | PKS-I            |
| Berg02.8.1| alpha proteobacterium CRA 3GB; AY562562 | Pseudovibrio     | PKS-I            |
| Berg02.8.3| sponge bacterium Isolate1; AY948382 | Pseudovibrio     | N/D              |
| Berg02.9.1| alpha proteobacterium CRA 3GB; AY562562 | Pseudovibrio     | N/D              |
| Berg02.36 | alpha proteobacterium CRA 3GB; AY562562 | Pseudovibrio     | N/D              |
| Berg02.39.1| sponge bacterium Isolate3; AY948383 | Pseudovibrio     | NRPS/PKS-I       |
| Berg02.39.3| sponge bacterium Isolate1; AY948382 | Pseudovibrio     | NRPS/PKS-I       |
| Berg02.40 | sponge bacterium Isolate1; AY948382 | Pseudovibrio     | PKS-I            |
| Berg02.61 | Pseudovibrio ascidicacecola (T); F423(= NBRC 100514); AB175663 | Pseudovibrio     | PKS-I            |
| Berg02.63 | sponge bacterium Isolate1; AY948382 | Pseudovibrio     | N/D              |
| Berg02.65 | Pseudovibrio ascidicacecola (T); F423(= NBRC 100514); AB175663 | Pseudovibrio     | N/D              |
| Berg02.141| sponge bacterium Isolate1; AY948382 | Pseudovibrio     | PKS-I            |
| Berg02.188| sponge bacterium Isolate3; AY948383 | Pseudovibrio     | N/D              |
| Berg01.114| Bacillus sp. enrichment culture clone SYW22; FJ601652 | Bacillus         | NRPS            |
| Berg02.161a| Bacillus sp. R5654(2010); GU968484 | Bacillus         | NRPS            |
| Berg02.117| Sporosarcina luteola       | Sporosarcina     | N/D              |
| Berg02.77 | Pseudomonas sp. C111064; AF500211 | Pseudomonas      | NRPS            |
| Berg02.64.2| Vibrio sp. Mel 35; AJ582806 | Vibrio           | PKS-I            |
| Berg02.104a| Vibrio cassinettae; LGP R; AJ582809 | Vibrio           | PKS-I            |
| Berg02.105a| Vibrio sp. Mel 35; AJ582806 | Vibrio           | PKS-I            |

Bacteria that did not amplify for any of the genes are not shown.
N/D – not determined.
doi:10.1371/journal.pone.0078992.t003
metabolites fundamental for sponge protection against other organisms. These communities have thus a great biotechnological importance in the search for new and more effective pharmacological drugs needed for the treatment of severe human diseases such as cancer, microbial infections and inflammatory processes. Our results evidenced the bioactive potential of the heterotrophic bacterial community of the sponge *Erylus discophorus* and open the way for further studies.

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**Author Contributions**

Conceived and designed the experiments: OML APG JB HG FV. Performed the experiments: APG JB MCM MC DOC. Analyzed the data: APG JB MCM MC DOC FV OML. Contributed reagents/materials/analysis tools: JB HG JRX FV OML. Wrote the paper: OML APG JB HG JRX FV DOC.

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