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Culturable Heterotrophic Bacteria Associated with Healthy and Bleached Scleractinian *Madracis decactis* and the Fireworm *Hermodice carunculata* from the Remote St. Peter and St. Paul Archipelago, Brazil

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Abstract We report on the first characterization of the culturable heterotrophic bacteria of the scleractinian *Madracis decactis*. In addition, we characterized the culturable bacteria associated with the fireworm *Hermodice carunculata*, observed predating partially bleached coral colonies. Our study was carried out in the remote St. Peter and St. Paul Archipelago (SPSPA), Mid-Atlantic Ridge, Brazil. We constituted a 403 isolates collection and subsequently characterized it by means of *pyrH* and 16S rRNA partial sequences. We identified *Photobacterium*, *Bacillus*, and *Vibrio* species as members of the culturable microbiota of healthy *M. decactis*. *V. campbellii*, *V. harveyi*, *V. communis*, and *V. maritimus* were the most commonly found *Vibrio* species in healthy corals, representing more than 60 % of all vibrio isolates. Most of the vibrios isolated from the fireworm's tissues ($n = 143$; >90 %) were identified as *V. shiloi*. However, we did not recover *V. shiloi* from bleached *M. decactis*. Instead, we isolated *V. communis*, a novel *Photobacterium* species, *Bacillus*, *Kocuria*, and *Pseudovibrio*, suggesting a possible role of other facultative anaerobic bacteria and/or environmental features (such as water quality) in the onset of bleaching in SPSPA's *M. decactis*.

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Introduction

The coral holobiont comprises the coral host and its symbiotic microbiota [44]. The microbiota is a complex community of eukaryotic microbes (e.g., zooxanthellae, microalgae, and fungi), prokaryotes, and viruses, representing a vast functional and genetic diversity. It became evident that corals harbor an enormous diversity of bacteria, both well-known cultivable taxa (e.g., vibrios) and novel groups (e.g., [12, 38]) awaiting formal taxonomic characterization. Vibrios appear as a considerable fraction of the microbiome of different coral species (with counts of up to 10^7 cells mL^{-1} in the coral mucus), in both healthy [2, 14, 23, 25] and diseased specimens [9, 22, 57]. Based on high vibrio colony counts and a high proportion of vibrio-related genes (e.g., *N*-acetylglucosaminases) in coral reefs, some authors suggested that an enrichment of vibrios in coral mucus and in the surrounding waters might indicate unhealthy environmental conditions [10, 16]. Vibrios may account for 38 % of the microbiota in *Pocillopora damicornis* [8], and up to 68 % in *Oculina patagonica* [23]. However, the role of these microorganisms in the coral

holobiont seems to be controversial. Claimed beneficial effects include nitrogen fixation [13], food resource [22], chitin decomposition [17], and production of antimicrobials [42]. On the other hand, *V. shiloi* (AK1^T) and *V. coralliilyticus* (YB1^T) were described and characterized as etiological agents of coral bleaching [7, 24, 44]. The majority of vibrios isolated from the corals *Mussismilia braziliensis*, *M. hispida*, and *Phyllogorgia dillatata* and from the zoanthids *Palythoa caribaeorum*, *P. variabilis*, and *Zoanthus solanderi* fell within the vibrio core group (*V. harveyi*, *V. communis*, *V. rotiferianus*, *V. campbellii*, and *V. alginolyticus*) according to *pyrH* gene sequences [2, 14, 15]. However, these previous studies were carried out in coastal regions where disease (and bleaching) is usually linked to anthropogenic impacts such as poor water quality [28, 50]. This study was performed in St. Peter and St. Paul Archipelago (SPSPA), one of the smallest and most isolated oceanic systems in the world. It is constituted by a group of ten islets near the axis of the Mid-Atlantic Ridge, approximately 1,000 km off the northeastern Brazilian coast and 1,900 km south-west of Senegal, between the northern and southern hemispheres [19] (Fig. 1). The analysis of the diversity of vibrios associated with corals from extremely isolated areas, with little local impacts, would offer an excellent opportunity to unravel the diversity of the culturable heterotrophic microbiota. The zooxanthellate scleractinian corals *Madracis decactis* and *Scolymia wellsi*; and the black corals *Tanacetipathes thamnea* and *T. hirta* are most commonly found in SPSPA. All of them are restricted to depths below 20 m and most abundant in depths below 40 m ([18, 19]; R. B. Francini-Filho, unpublished data) where water temperature is less likely to follow occasional superficial seawater rises. Lower depths ($\sim$5 m) are dominated by the zoanthid *P. caribaeorum.* Another important feature of the study area is the presence of the fireworm *Hermodice carunculata* [1], a well-known corallivore, showing preference toward juveniles and larvae [31, 37, 58]. This polychaete is the winter reservoir and summer vector of *V. shiloi* in the Israeli Mediterranean [49]. However, it is not yet clear if fireworms from other geographic regions harbor this bacterium. *M. decactis* and *H. carunculata* both appear in different continents, but little is known about their associated microbiotas. *M. decactis* displays a wide distribution along the Brazilian and Caribbean coasts (from Parcel de Manuel Luiz Reefs, in the north, to the coast of Santa Catarina, in the south) and occurs in all Brazilian Oceanic Islands (Fernando de Noronha, Roca Atol, SPSPA, and Trindade/Martim Vaz) [11, 34, 35, 39] and West Africa [35]. *M. decactis* accounts for relevant reef structures in southeastern Brazil (São Paulo) and these populations were seriously affected by a bleaching event during 1993/1994 summer [32]. Our aim was to analyze the diversity of heterotrophic bacteria (vibrios) associated with *M. decactis* and *H. carunculata* in SPSPA. In addition, we evaluated environmental parameters (water quality) and the pathogenic potential of a subset of representative vibrio isolates.

Materials and Methods

Study Area

This survey took place in depths between 20 and 30 m at SPSPA, which is constituted by the emerged summits of the Mid-Atlantic Ridge (Fig. 1). On the biggest islet, Belmont, lies the Scientific Station built and maintained by the Brazilian Navy. Altogether the islets enclose a U-like bay measuring approximately 100 $\times$ 50 m (Fig. 1), where occasional and intermittent upwelling has been suggested to correlate to water turbulence [3]. Depths in the bay vary from 6 to 35 m, dropping abruptly to reach much deeper plateaus (700–900 m) and the abyssal plain underneath (3,600–5,000 m) [29, 33]. Water visibility is rarely less than 15 m and surface seawater temperature ranges from 26 to 29 °C according to the Prediction and Research Moored Array in the Atlantic database—PIRATA (www.pmel.noaa.gov/pirata/display.html).

Field Sampling

Sampling was made on 4 days of September/2010 along the inner bay and adjacent walls by means of SCUBA diving. The sites and sampling days were: Site 1: days 14 and 15; and Site 2 (almost outside the inlet): days 18 and 22. Distance among sites was less than 100 m (00°56′N; 29°22′W) (Fig. 1). In total, 12 colony fragments (10 $\times$ 10 cm) of *M. decactis* (healthy: $n = 8$; bleached: $n = 4$) and three specimens of *H. carunculata* were sampled. Coral fragments were removed with a hammer and chisel. All samples were placed in plastic bottles underwater including approximately 200 mL of seawater. Four colony fragments of healthy *M. decactis* were collected from site 1: 14 ($n = 2$) and 15 ($n = 2$). Bleached ($n = 4$) and healthy ($n = 4$) coral fragments were collected from site 2: 18 (healthy: $n = 2$; bleached: $n = 2$) and 22 (healthy: $n = 2$; bleached: $n = 2$). Fireworms were collected from Site 2 (locale of *M. decactis*' colonies predation). Seawater from the water column immediately above the corals (<1 m) was sampled (4 samples, 20 L/sample) for vibrio and total bacterial counts plus chemical analysis. Field observations were documented by photo and video (Nikon D90 with Aquatica housing). All samples were taken immediately ($\sim$5 min) from the pier (Fig. 1), where the divers emerged, to the Scientific Station Laboratory ($\sim$20 m distant), processed and preserved properly for further analysis.

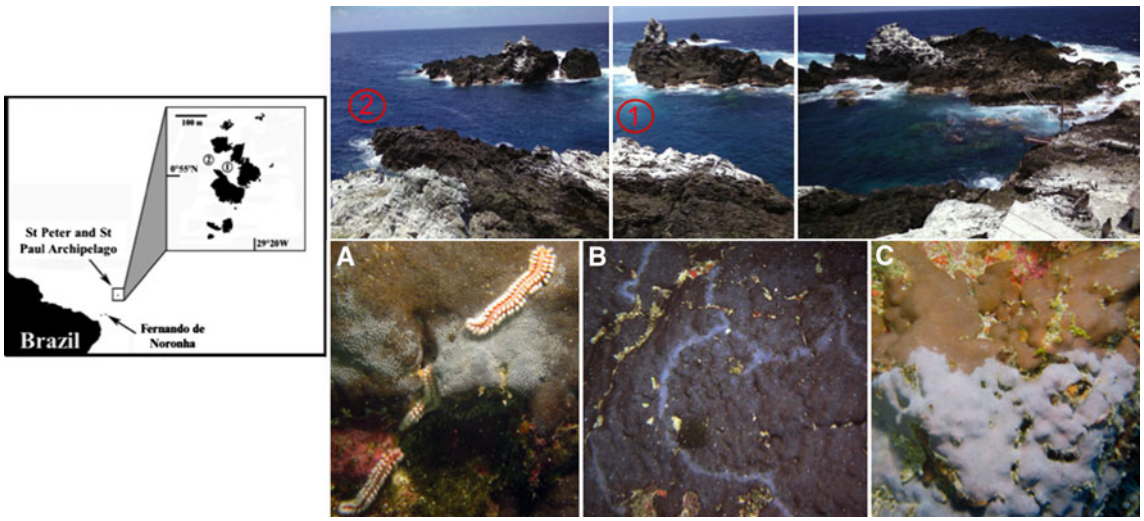

Fig. 1 *Left* map showing SPSPA location. *Right-top* view of the SPSPA inner bay, where sampling Sites 1 and 2 are *indicated* (*photos* A. P. B. Moreira). *Right-bottom* **a** colony of *M. decactis* infested with fireworms (note bleached tissue). **b** Dead and bleached tissue along the fireworm's paths on *M. decactis*. **c** Colony of *M. decactis* after infestation by the fireworms (*photos* R. B. Francini-Filho)

Seawater Physical–Chemical and Biological Measurements

Seawater temperature was measured at the moment and location of sampling by using a portable thermometer (Hobo, ONSET Corporation). Salinity, inorganic nutrients, and microbial abundance were measured as described previously [10]. At least three replicates were analyzed for each parameter.

Isolation, Preservation, and Enumeration of Bacteria

Coral and fireworms samples were immediately rinsed three times with 50 mL sterile saline solution (3 % NaCl, SSS) to remove loosely associated microorganisms from the water column and particles. Culturing was performed for seawater samples (enumeration only) and for corals and fireworm tissues (enumeration plus isolation) at the SPSPA. Approximately 1 cm^3 of coral tissue was detached from the coral skeleton by scraping and crushing. Fireworms were also crushed in order to provide approximately the same volume of tissue (1 cm^3). The particulate material was transferred to sterile tubes with SSS (10 mL) and resuspended by vigorous vortex for 3 min. After 15 min of sedimentation, supernatant aliquots (1 mL) were 10-fold serial diluted in SSS and plated onto Marine Agar (MA, Difco) and on vibrio-selective (but not vibrio-specific) thiosulfate–citrate–bile salt–sucrose agar [21] (TCBS, Oxoid) in triplicates. Sub-samples (0.1 mL) of seawater from each site were inoculated on three plates (TCBS). Colony forming units (CFUs) on TCBS (putative Vibrio CFUs) were enumerated after 24 h incubation (TCBS) at ambient temperature (27–28 °C). The error measure for counts was standard deviation. On MA growth was followed for 48 h–1 week. At least 20 representative colony morphotypes from each of the 15 specimens were picked (MA and TCBS) from the highest dilution (10^4–10^6), and transferred to 96-well plates with semi-solid MA (0.8 % agar) for further purification at UFRJ. Approximately 10 % of the cultures did not grow on sub-culture. Pure cultures were preserved in cryovials with glycerol (20 %) at −80 °C.

Taxonomic Characterization

Microbial diversity was characterized by partial 16S rRNA and *pyrH* gene sequencing of putative heterotrophic bacteria (retrieved from MA) and putative vibrios (retrieved from TCBS agar), respectively, as described previously [14, 52, 54], with modifications as follows. DNA was extracted either by boiling lysis or through Pitcher et al.'s methodology [40]. PCR products were purified with ExoSAP-IT® (Affymetrix) and underwent the sequencing reactions with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems), following the manufacturer's instructions. Separation of the DNA fragments was performed at Genetic Analyzer® model 3500 (Applied Biosystems). Primers are listed in Online Resource 1. Raw sequence data were transferred to the Gene Builder module, Kodon package 2.03 (Applied Maths, Belgium) or ChromasPro 1.7.1 (Technelysium Pty. Ltd, Tewantin, Australia) where consensus sequences were determined. ClustalW was employed for sequence alignment. Similarity matrices and phylogenetic trees were constructed using MEGA 5.0 [51]. Trees were reconstructed using the Neighbor-Joining method [46]. The

evolutionary distances were computed using the Jukes–Cantor method [20]. The robustness of each topology was checked by a 1,000 bootstrap replications. *pyrH* gene alleles were determined through the MLST Data Analysis: non-redundant databases—NRDB (http://pubmlst.org/perl/mlstanalyse/mlstanalyse.pl?site=pubmlst&page=nrdb&referer=pubmlst.org). GenBank Accession numbers for the gene sequence data generated in this study are KC751008–KC751092 (16S rRNA) and KC751093–KC751342 (*pyrH*). The gene sequence data are also available through our website TAXVIBRIO (http://www.taxvibrio.lncc.br/).

Pathogenicity Tests

Artemia nauplii was used as the animal model as described previously [4, 47], with modifications, as follows. For essays, *A.* nauplii were placed in each well of a 24-well plate with *V. shiloi* cells (10^8 or 10^9 CFUs mL^{-1}) within 2.5 mL of sterile seawater. Each replicate included 30 *A.* nauplii individuals. Experiments were performed at least in quadruplicates and repeated at least three times, at 30 °C for 48 h. Differences among samples and controls were evaluated by using Analyses of Variance followed by Tukey's Multiple Comparison Test. Differences were considered significant when $P < 0.05$.

Results

High densities of fireworms ($n = 20$ colonies; 25 % of them containing fireworms; Fig. 1) were observed infesting *M. decactis* at Site 2, where temperature variation was 22–24 °C (Table 1). Bleaching was observed in all infested colonies, while no signs of bleaching was observed in non-infested ones (Fig. 1). In Site 1, where colonies were not infested, there were no signs of bleaching either.

Environmental Parameters, Total Bacterial and Vibrio Counts

Temperature was lower (<25 °C) at Site 2 compared to Site 1 (26–27 °C; Table 1). Inorganic nutrients, total bacterial and seawater vibrio counts showed a rising pattern along the sampling period. The highest values for orthophosphate, total phosphorous, and ammonia were on the 15th, for nitrite on the 18th, and for nitrate and total nitrogen (>6 μM) on the 22nd (the lowest values were on the 14th for all these parameters). Total bacterial counts in seawater were highest on the 22nd and vibrio counts in seawater were lower than 100 CFU ml^{-1}, with the highest value on the 18th (75 ± 35; $n = 3$). Vibrio counts (CFU cm^{-3}) in *M. decactis* tissues varied between 3.85E+04 (±5.44E+04; $n = 2$) and 2.63E+06

Table 1 General features of sampling sites in SPSPA and samples

	Site 1		Site 2	
Geographic location	00°56′N; 29°22′W			
Date	14 September 2010	15 September 2010	18 September 2010	22 September 2010
Depth (m)	<30			
Temperature (°C)	26–27		22–24	
Salinity (S)	35.85 ± 0.0428	35.9 ± 0.0236	35.94 ± 0.0035	35.88 ± 0.004
Silicate (μmol L^{-1})	0.34 ± 0.00	0.35 ± 0.038	0.32 ± 0.0153	0.36 ± 0.058
Orthophosphate (μmol L^{-1})	0.06 ± 0.005	0.38 ± 0.0073	0.11 ± 0.0063	0.13 ± 0.011
DIP (μmol L^{-1})	0.09 ± 0.00	0.44 ± 0.055	0.15 ± 0.01	0.15 ± 0.00
Ammonia (μmol L^{-1})	0.16 ± 0.0265	0.67 ± 0.04	0.18 ± 0.0208	0.19 ± 0.055
Nitrite (μmol L^{-1})	0.03 ± 0.0058	0.007 ± 0.0058	0.06 ± 0.0058	0.03 ± 0.00
Nitrate (μmol L^{-1})	0.16 ± 0.04	<0.074	0.19 ± 0.0802	0.25 ± 0.0473
DIN (μmol L^{-1})	2.81 ± 0.4950	5.40 ± 0.5311	6.09 ± 0.1980	6.27 ± 0.1131
Bacterial counts (cells mL^{-1})	ND	ND	4.80E+05 ± 9.49E+04	3.14E+06 ± 1.42E+06
Vibrio counts (CFU mL^{-1})				
Water	10 ± 17.3	16.7 ± 15.3	75 ± 35.4	26.7 ± 15.3
Madracis decactis	2.63E+06 ± 1.02E + 06	ND	ND	3.85E+04 ± 5.44E+04
Madracis decactis (Ble)	ND	ND	ND	2.33E+03 ± 2.31E+03
Hermodice carunculata	ND	ND	ND	1.35E+04 ± 4.95E+03

Mean ± standard errors of three independent measurements. Vibrio counts for coral tissue were performed in duplicate

DIP dissolved inorganic phosphorous (total phosphorous), *DIN* dissolved inorganic nitrogen (total nitrogen) *Ble* bleached, *ND* not determined

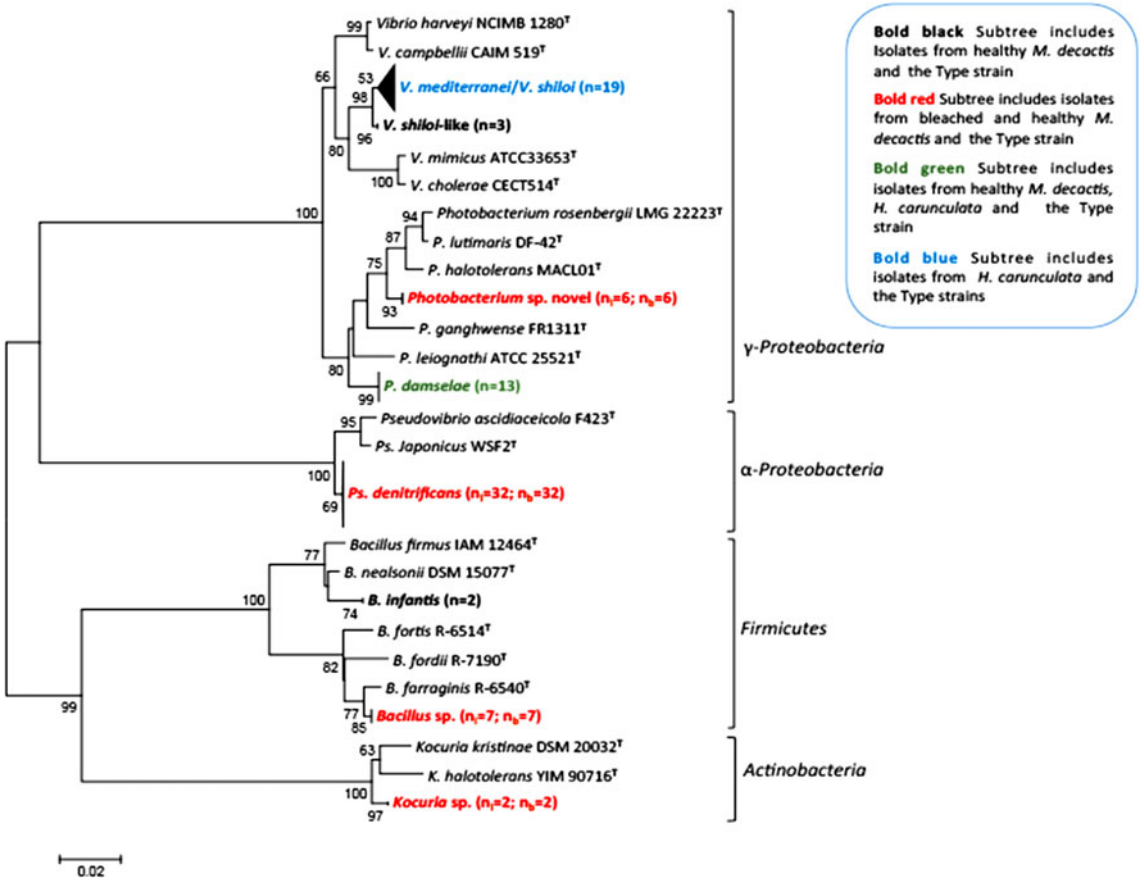

Fig. 2 Diversity of heterotrophic bacteria from the fireworm *H. carunculata*, healthy and bleached *M. decactis*. Isolates groups and the corresponding type species are compressed for better visualization of the entire tree, where n_i indicates the number of representative isolates included in the subgroup. The species groups that include strains retrieved from bleached *M. decactis* are also informed following n_b. Isolates of a novel species of *Photobacterium* were retrieved from bleached *M. decactis*. Analysis included 109 sequences (84 representative isolates; 25 type strains) and 810 nucleotide positions. *Scale bar* indicates 2 % nt divergence. Bootstrap values of <50 % are not shown

(±1.02E+06; $n = 2$; on the 22nd). Bleached *M. decactis* presented lower counts (2.33E+03 ± 2.31E+03; $n = 2$; on the 22nd). Vibrio counts reached 1.35E+04 (±4.95E+03; $n = 3$; on the 22nd) in the fireworm's tissues (Table 1). It is worth to register that sharp tidal oscillations accompanied by occasional vortex intensified as full moon approached (23 September 2010).

Heterotrophic Bacterial Diversity

A collection of 403 bacterial isolates was established, whereof 325 were retrieved from *M. decactis* and *H. carunculata* (Figs. 2, 3; Online Resources 2, 3). Approximately 80 % of the isolates retrieved from *M. decactis* and the fireworms belonged to the genus *Vibrio* ($n = 259$, 205 retrieved from TCBS; Fig. 3). A higher diversity of vibrios (*V. campbellii*, *V. communis*, *V. harveyi*, *V. maritimus*, *V. pelagius*, *V. chagasii*, *V. ponticus*, *V. tubiashii*, and two potential new species) was observed in healthy *M. decactis* in comparison with bleached corals, from which only *V. communis* ($n = 4$ strains: A-290–293) was retrieved (Fig. 3). Strain A-290 was identical to A-335 (based on *pyrH* gene sequences; Online Resource 4), which was isolated from healthy *M. decactis* of Site 1. Other heterotrophs from *M. decactis* were *Photobacterium angustum*, *Bacillus infantis* (healthy), and members of *Photobacterium* (a novel species), *Bacillus*, *Kocuria,* and *Pseudovibrio* (*Ps. denitrificans*) (bleached; Fig. 2). Most of the isolates (>90 %) retrieved from *H. carunculata* were identified as *V. shiloi* ($n = 143$ strains out of 157; Fig. 3). *V. shiloi* A-328, A-354, and A-384 were obtained from healthy *M. decactis* (Fig. 3; Online Resource 5). *V. shiloi* from SPSPA comprised at least 31 populations, according to the analysis of *pyrH* alleles (Online Resource 5). No *V. shiloi* was recovered from bleached *M. decactis*.

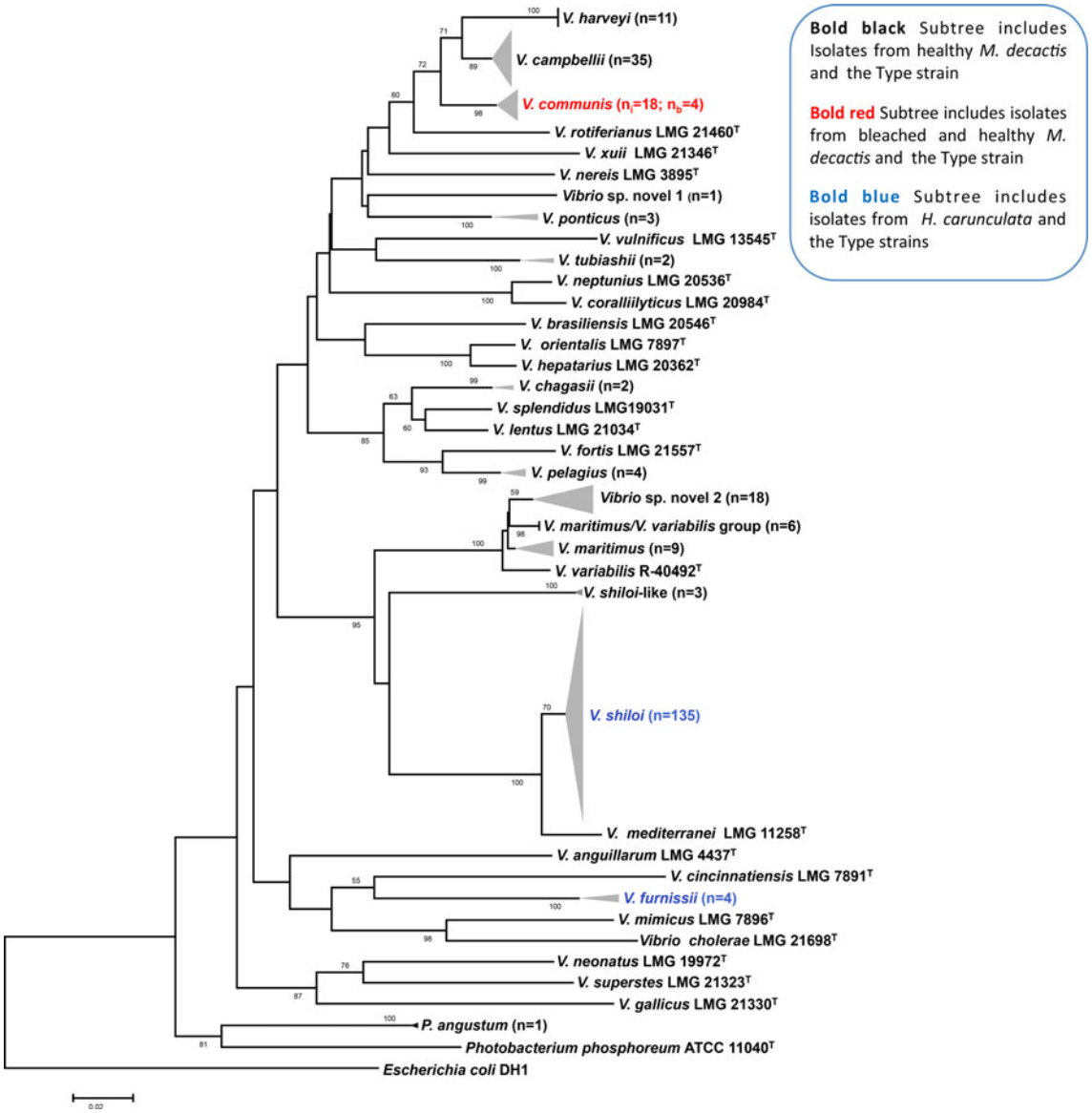

Fig. 3 Diversity of vibrios retrieved from the fireworm *H. carunculata*, healthy and bleached *M. decactis* from SPSPA. Isolates groups and the corresponding type species are compressed for better visualization of the entire tree, where *n* indicates the number of isolates included in the subgroup. For *V. communis* group, n_i indicates the total number of isolates and the following n_b indicates the number of strains retrieved from bleached *M. decactis*. Potential new *Vibrio* species were retrieved from healthy *M. decactis.* At least 14 *Vibrio* species were found, including 2 potential new species. Analysis included 286 sequences (251 representative isolates; 35 type strains) and 429 nucleotide positions. *Scale bar* indicates 2 % nt divergence. Bootstrap values of <50 % are not shown

Pathogenic Potential of *V. shiloi* Strains

Strains A-16, A-180, A-203 (from *H. carunculata*), and A-328, A-354, A-384 (from healthy *M. decactis*) were chosen as representatives of *V. shiloi* populations (Online Resource 5). Two isolates, A-354 and A-384 (*M. decactis*), killed *A.* nauplii, with average 40 % mortality after 48 h compared to controls without bacteria (Fig. 4). Both isolates promoted equivalent effect, which was significantly different from controls ($P < 0.0001$).

Discussion

Our study reports on the heterotrophic (vibrio) diversity associated with *H. carunculata* and *M. decactis* in one of the smallest and most isolated archipelagos in the world. We observed *Photobacterium*, *Pseudovibrio*, *Bacillus*, and *Kocuria* species as members of the culturable microbiota of *M. decactis*. All these genera have been reported not only as members of corals' regular microbiota but also as coral disease associated groups ([2, 25, 27, 30, 45, 53]; Bondarev

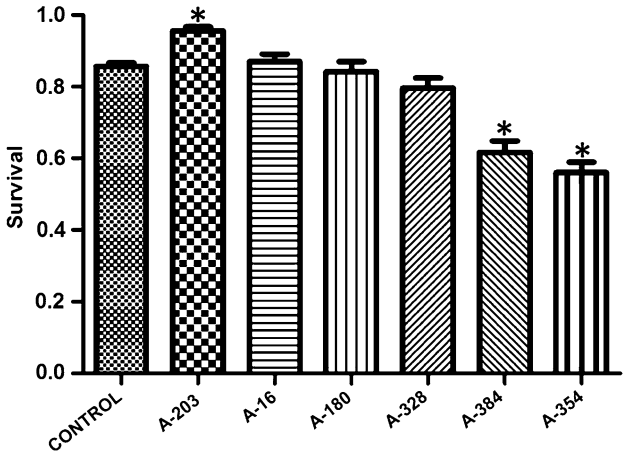

Fig. 4 Pathogenic potential of *V. shiloi* A-203, A-16, A-180 (from *H. carunculata*); A-354, A-384 and A-328 (from *M. decactis*). Bioessays using *A.* nauplii. Strains A-354 and A-384 reduced survival. Bacterial treatments contain 10^8 CFUs mL^{-1}. *Asterisks* indicate results significantly different from control (without bacteria)

et al., direct submission—NCBI accession NC_016642). *V. campbellii*, *V. harveyi*, *V. communis*, and *V. maritimus* were the most commonly found *Vibrio* species in healthy corals, representing more than 60 % of all vibrio isolates. We did not recover *V. shiloi* from bleached *M. decactis*, despite the infestation by fireworms, which were harboring this bacterium. These findings refer to previous works where it was demonstrated that *V. shiloi* from fireworms may adhere to corals due to chemotaxis, but in less than 72 h after penetrating the coral cells it enters a viable-but-not-culturable [36] state [5, 55]. Instead, we isolated four strains of *V. communis* (also retrieved from healthy corals), a novel *Photobacterium* species (under characterization), and *Ps. denitrificans*, suggesting a possible role of other bacteria and anaerobic processes in bleached corals [48]. During the survey the maximum seawater temperature was 27 °C at Site 1 and 24 °C at Site 2, location where colonies were bleached, suggesting that temperature might not have triggered the bleaching process. On the other hand, inorganic nutrients (nitrogen) in seawater might have selectively favored the growth of microbes (e.g., *Ps. denitrificans*) in bleached corals of Site 2. For instance, dissolved inorganic nitrogen (DIN; >6 μM) was higher compared to Site 1 (2–5 μM; Table 1). Differences in nutrients among sites might reflect water turbulence, which intensified along the survey from the 14th (Site 1) toward the 22nd (Site 2). Nutrient levels recorded reached 6-fold of the suggested threshold concentration for DIN (1.0 μM) and twice that for dissolved inorganic phosphorous (DIP; 0.2 μM) determined for the Great Barrier Reef [6]. These thresholds were reported to promote the onset of eutrophication also in other reefs (e.g., Barbados and the Florida Keys) [26, 56]. Whether a natural temporary eutrophication process occurred with reflex upon the coral's microbiota are issues to be deeper investigated. Nevertheless the observations of bleached *M. decactis* as a result of a possibly temperature-independent process and that *H. carunculata* is a reservoir of *V. shiloi* in SPSPA are relevant findings that might contribute for the understanding of bleaching in this far-off site. *V. shiloi* A-354 and A-384 killed *A.* nauplii whereas other representative isolates did not, suggesting that the populations of *V. shiloi* from SPSPA have different virulence levels and repertoires. A-354 and A-384 might display an even more versatile armory than *V. shiloi* $AK1^T$, which was not capable of causing mortality to artemias [4]. $AK1^T$ pathogenomics [41] confirmed the presence of the genes required to cause coral bleaching according to the *V. shiloi*/*O. patagonica* model of coral bleaching previously proposed [43]. In this context, the genomic comparison of strains A-354 and A-203 (which promoted opposite effects against artemias, Fig. 4) with $AK1^T$ should be informative. This study was a first attempt to characterize the heterotrophic culturable bacterial diversity in the remote SPSPA. The coral *M. decactis* and the fireworm *H. carunculata* are reservoirs of vibrios (including *V. shiloi*) and other quimiorganotrophic facultative anaerobic bacteria, some of which may be detrimental to the coral holobiont homeostasis. Our study also points out the broad role of the microbiota in the health of *M. decactis*. Bleaching in *M. decactis* is a complex phenomenon possibly influenced by the physical–chemical and (micro)biological features of SPSPA.

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