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Vibrios dominate as culturable nitrogen-fixing bacteria of the Brazilian coral *Mussismilia hispida*

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Abstract

Taxonomic characterization was performed on the putative N_2-fixing microbiota associated with the coral species *Mussismilia hispida*, and with its sympatric species *Palythoa caribaeorum*, *P. variabilis*, and *Zoanthus solanderi*, off the coast of São Sebastião (São Paulo State, Brazil). The 95 isolates belonged to the *Gammaproteobacteria* according to the 16S rDNA gene sequences. In order to identify the isolates unambiguously, *pyrH* gene sequencing was carried out. The majority of the isolates ($n = 76$) fell within the *Vibrio* core group, with the highest gene sequence similarity being towards *Vibrio harveyi* and *Vibrio alginolyticus*. Nineteen representative isolates belonging to *V. harveyi* ($n = 7$), *V. alginolyticus* ($n = 8$), *V. campbellii* ($n = 3$), and *V. parahaemolyticus* ($n = 1$) were capable of growing six successive times in nitrogen-free medium and some of them showed strong nitrogenase activity by means of the acetylene reduction assay (ARA). It was concluded that nitrogen fixation is a common phenotypic trait among *Vibrio* species of the core group. The fact that different *Vibrio* species can fix N_2 might explain why they are so abundant in the mucus of different coral species.

Keywords: N_2-fixing bacteria; Vibrios; *V. alginolyticus*; *V. harveyi*; Coral; *Mussismilia hispida*; *Palythoa caribaeorum*; *P. variabilis*; *Zoanthus solanderi*

Introduction

Coral reefs are among the most productive and diverse ecosystems within coastal tropical environments, mainly in oligotrophic regions [9]. All the coral reefs of the South Atlantic Ocean are spread throughout the northeastern coast and continental shelf of Brazil. The diversity of coral fauna is low, and mainly consists of relics from the Tertiary period. Brazilian coral reefs show initial growth as a mushroom-like structure, with a considerable amount of incrusting coralline algae [15,14]. Their fauna is composed mainly of cnidarians from the class *Anthozoa*, order *Scleractinia*, family *Mussidae*, and genus *Mussismilia* [6].

Mussismilia hispida is one of the seven scleractinian species and it has the widest geographic distribution. It inhabits from Santa Catarina to Rio Grande do Norte

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(ca. 5000 km), which indicates its adaptation to wide environmental gradients, such as temperature, water turbidity and pollution. *M. hispida* is endemic to Brazil, and one of the major reef-builders along the north-eastern Brazilian coast. The Brazilian corals of the genus *Mussismilia* are in danger of extinction [11], possibly due to a variety of stressors, including infection. However, so far, no data is available on the taxonomic composition of the microbiota of *M. hispida*.

Corals and coral reefs have experienced a tremendous decline in recent decades. Global warming, pollution, and infectious diseases, particularly those caused by vibrios, are among the main causes of the increasing stress that they are suffering worldwide [10,13,32]. Culture-independent studies based on 16S rDNA clone libraries and metagenomics have shown that vibrios are abundant in the mucus of different coral species, with significant increases in vibrio populations immediately before massive bleaching events, leading to a dominance of vibrio sequences in the sequence libraries [4,5]. Indeed, *Vibrio alginolyticus*, *V. coralliilyticus*, *V. harveyi*, and *V. shilonii* (= *V. mediterranei*) have been shown to be coral pathogens [12,19,26] and are found in association with different coral species [4,18].

On the other hand, some vibrios may also establish mutualistic partnerships with corals by providing nutrients and secondary metabolites (e.g. bacteriocins) to their hosts [18]. Corals may harbor a variety of N_2-fixers which may provide a substantial amount of the total nitrogen needed by the host metabolism [16,23,31]. Nitrogen fixation, the production of NH_3 by the reduction of N_2, is carried out by nitrogenases and is a tightly regulated process under the control of the NtrC activator protein. This protein is a response regulator and its phosphorylated form will induce transcription of nitrogenases when NH_3 is not available. Coral reefs occur in oligotrophic areas possibly because of the N_2 fixation activity occurring in the corals themselves [7].

In the present study, taxonomic characterization was performed on the dominant culturable N_2-fixing microbiota associated with *M. hispida* and its sympatric species *Palythoa caribaeorum*, *P. variabilis*, and *Zoanthus solanderi*. The genera *Palythoa* and *Zoanthus* belong to the phylum *Cnidaria*, class *Anthozoa*, order *Zoanthidea* and family *Zoanthidae*, comprising shallow water zooxanthelate species [6]. The genera *Palythoa* and *Zoanthus* appear to be widespread in different continents. By examining these sympatric cnidarian species, the host specificity of the microbiota of each taxon was evaluated. In order to confirm that *V. alginolyticus*, *V. campbellii*, *V. harveyi*, and *V. parahaemolyticus* isolates were able to fix N_2, successive passages in nitrogen-free medium were undertaken and, subsequently, representative isolates were subjected to the acetylene reduction test.

Materials and methods

Thirty-two cnidarian specimens belonging to four species were collected on 3 February 2005 at three sites: Grande (23°50′25″S; 045°24′59″W), Portinho (23°50′25″S; 045°24′22″W) and Preta (23°49′10″S; 045°24′37″W) beaches located near the Centro de Biologia Marinha-USP (CEBIMAR-USP; São Sebastião Channel, São Paulo, Brazil) by SCUBA diving between depths of 3 and 7 m. The beaches Grande, Portinho and Preta are about 2 km apart from each other, the latter being on the continental side of the São Sebastião Channel and the first two are opposite facing CEBIMAR-USP. The cnidarian specimens were associated with rocky shores at these sites. Intact colonies of *M. hispida* and fragments of zoanthids were placed in sterile plastic bags and kept at ca. 10 °C for 6 h prior to microbiological examination. Samples were taken to the University of Campinas for isolation, purification, and characterization of the microorganisms.

Isolation and preservation of strains

The isolation of putative N_2 fixers from the cnidarian mucus was performed using the nitrogen-free (NFb) selective medium supplemented with 3% NaCl [2]. The mucus was drained from the coral samples using a sterile syringe. Tenfold dilutions of coral mucus were obtained in sterile saline solution (3% NaCl). A total of 100 ml aliquots of the dilutions were plated onto NFb and 2–8 representative colony morphotypes were picked for further purification from the highest dilution (10^4) after 4 days of incubation at 28 °C. Isolates obtained in this study are listed in Table 1. Pure cultures were maintained in vials with 20% glycerol at −80 °C.

Taxonomic characterization

The preliminary characterization of all pure cultures was obtained by 16S rDNA gene sequences, as described previously but with minor modifications [25]. The reactions were composed of 37.5 μl sterile MilliQ water, 5.0 μl PCR buffer (10 ×), 1.5 μl Mg_2Cl (1.5 mM), 0.4 μl dNTP's (0.2 mM each), 1 μl forward primer p27f (5′AGA GTT TGA TCM TGG CTC AG3′, 20 μM), 1 μl reverse primer (5′CGG TGT GTA CAA GGC CCG GGA ACG3′, 20 μM), 0.4 μl AmpliTaq DNA Polymerase (2 U/μl), and 1 μl template DNA (0.02 μg/μl). The thermal program consisted of (1) 2 min at 95 °C, (2) 30 cycles of 1 min at 94 °C + 1 min at 55 °C and 3 min at 72 °C, and (3) 3 min at 72 °C. PCR was performed using an Eppendorf thermocycler. The PCR products were purified using a solution of PEG8000 (20%)/2 M NaCl. Purified PCR products were eluted in 50 μl sterile MilliQ water. Subsequently, 5.0 μl of purified PCR product were mixed with 4.0 μl ET Terminator™ Mix

Table 1. Strain list

Species name	Strain no.	Colony morphotype	Source
V. alginolyticus	R-228	3.3.a	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-232	3.2.b	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	**R-234****	**1.16.d**	***Mussismilia hispida*, Praia Grande**
V. alginolyticus	**R-235****	**3.3.b**	***Zoanthus solanderi*, Praia Preta**
V. alginolyticus	**R-262****	**2.5.c1**	***Mussismilia hispida*, Praia Portinho**
V. alginolyticus	R-263	1.13.b	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-265****	**1.3.b1**	***Palythoa caribaeorum*, Praia Grande**
V. alginolyticus	R-283	3.2.a	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-284	3.4.b	*Mussismilia hispida*, Praia Preta
V. alginolyticus	R-287	1.11.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-288	1.1.c2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-289	1.15.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-290****	**2.4.b**	***Mussismilia hispida*, Praia Portinho**
V. alginolyticus	R-291	1.1.b1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-292	1.1.a1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-293	1.1.d2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-294	3.3.c	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-295	2.10.b	*Palythoa variabilis*, Praia Portinho
V. alginolyticus	R-296	2.3.	*Palythoa caribaeorum*, Praia Portinho
V. alginolyticus	R-297	1.1.a2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-298	2.2.a	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-299	1.2.b	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-300****	**1.4.c**	***Mussismilia hispida*, Praia Grande**
V. alginolyticus	R-301	2.9.a2	*Palythoa variabilis*, Praia Portinho
V. alginolyticus	**R-302****	**2.2.b**	***Mussismilia hispida*, Praia Portinho**
V. alginolyticus	R-303	2.5.c2	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-304	1.6.d	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-306	1.6.b	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-308	3.3.d	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-309	1.12.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	**R-310***	**1.15.b**	***Mussismilia hispida*, Praia Grande**
V. alginolyticus	R-312	1.10.b2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-313	2.8.a	*Palythoa variabilis*, Praia Portinho
V. alginolyticus	R-314	2.4.a	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-315	2.4.c	*Mussismilia hispida*, Praia Portinho
V. alginolyticus	R-316	2.6.c	*Palythoa caribaeorum*, Praia Portinho
V. alginolyticus	R-317	2.6.b	*Palythoa caribaeorum*, Praia Portinho
V. alginolyticus	R-318	1.1.c1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-319	1.3.b2	*Palythoa caribaeorum*, Praia Grande
V. alginolyticus	R-320	1.3.a	*Palythoa caribaeorum*, Praia Grande
V. alginolyticus	R-321	1.14.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-322	1.4.c	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-323	1.1.b2	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-324	1.1.d1	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-325	1.8.a	*Palythoa caribaeorum*, Praia Grande
V. alginolyticus	R-326	1.6.a	*Mussismilia hispida*, Praia Grande
V. alginolyticus	R-329	3.3.e	*Zoanthus solanderi*, Praia Preta
V. alginolyticus	R-331	2.8.b	*Palythoa variabilis*, Praia Portinho
V. fortis	R-248	3.4.c	*Mussismilia hispida*, Praia Preta
V. fortis	R-254	1.11.b	*Mussismilia hispida*, Praia Grande
V. harveyi	**R-227****	**1.12.b**	***Mussismilia hispida*, Praia Grande**
V. harveyi	**R-230****	**1.5.c**	***Palythoa caribaeorum*, Praia Grande**
V. harveyi	**R-233***	**1.16.d**	***Mussismilia hispida*, Praia Grande**
V. harveyi	**R-239****	**1.6.c**	***Mussismilia hispida*, Praia Grande**
V. harveyi	R-242	1.7.a	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-243	2.7.a	*Palythoa caribaeorum*, Praia Portinho

Table 1. (*continued*)

Species name	Strain no.	Colony morphotype	Source
V. harveyi	**R-246****	**1.2.c**	***Mussismilia hispida*, Praia Grande**
V. harveyi	**R-253****	**1.11.c**	***Mussismilia hispida*, Praia Grande**
V. harveyi	R-255	3.5.a	*Palythoa caribaeorum*, Praia Preta
V. harveyi	R-257	2.7.c	*Palythoa caribaeorum*, Praia Portinho
V. harveyi	R-259	3.5.b	*Palythoa caribaeorum*, Praia Preta
V. harveyi	R-260	3.5.c	*Palythoa caribaeorum*, Praia Preta
V. harveyi	**R-264***	**1.4.a**	***Mussismilia hispida*, Praia Grande**
V. harveyi	R-280	2.9.a1	*Palythoa variabilis*, Praia Portinho
V. harveyi	R-285	2.7.b	*Palythoa caribaeorum*, Praia Portinho
V. harveyi	R-286	2.6.a	*Palythoa caribaeorum*, Praia Portinho
V. harveyi	R-305	1.9.c	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-307	1.8.b	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-311	3.6.a	*Palythoa caribaeorum*, Praia Preta
V. harveyi	R-327	1.9.b1	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-328	1.9.b2	*Palythoa caribaeorum*, Praia Grande
V. harveyi	R-330	1.8.c	*Palythoa caribaeorum*, Praia Grande
V. parahaemolyticus	**R-241****	**1.9.a**	***Palythoa caribaeorum*, Praia Grande**
V. tubiashii	R-229	1.5.b	*Palythoa caribaeorum*, Praia Grande
V. tubiashii	R-252	3.6.c	*Palythoa caribaeorum*, Praia Preta
Vibrio sp.	R-240	1.7.b	*Palythoa caribaeorum*, Praia Grande
Alteromonas sp.	R-250	1.16.c	*Mussismilia hispida*, Praia Grande
Alteromonas sp.	R-251	3.6.b	*Palythoa caribaeorum*, Praia Preta
Marinomonas sp.	R-236	1.14.d	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-237	1.14.d	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-238	3.1.	*Mussismilia hispida*, Praia Preta
Marinomonas sp.	R-247	3.4.d	*Mussismilia hispida*, Praia Preta
Marinomonas sp.	R-249	1.16.a	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-256	3.4.a	*Mussismilia hispida*, Praia Preta
Marinomonas sp.	R-261	1.15.c	*Mussismilia hispida*, Praia Grande
Marinomonas sp.	R-278	1.13.a	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-231	1.15.d	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-244	2.5.a	*Mussismilia hispida*, Praia Portinho
Pseudoalteromonas sp.	R-245	1.4.b	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-258	2.10.a	*Palythoa variabilis*, Praia Portinho
Pseudoalteromonas sp.	R-266	1.2.a	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-277	1.10.a	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-279	1.10.b1	*Mussismilia hispida*, Praia Grande
Pseudoalteromonas sp.	R-281	2.9.b	*Palythoa variabilis*, Praia Portinho
Pseudoalteromonas sp.	R-282	2.5.b	*Mussismilia hispida*, Praia Portinho

Representative strains tested for nitrogenase activity are indicated in bold.
The code used for the colony morphotypes is composed of three digits. The location (1, Praia Grande; 2, Praia Portinho; and 3, Praia Preta), specimen number (16 specimens from Praia Grande, 10 specimens from Praia Portinho, and six specimens from Praia Preta), and the colony type (a, b, c, onwards).
*Indicates growth after six successive passages in the minimal medium NFb.
**Denotes isolates with both phenotypes positive in the ARA test and growth after six successive passages in the minimal medium NFb.

(GE Health Care), 0.2 µl sequencing primer 782*r* (5′ACC AGG GTA TCT AAT CCT GT3′, 20 µM), and 0.8 µl MilliQ water. The thermal program consisted of 30 cycles of 20 s at 95 °C + 15 s at 50 °C + 1 min at 60 °C. The purification of the sequencing products was obtained by mixing 1 µl ammonium acetate (7.5 M) and 27.5 µl absolute ethanol, followed by incubation in the dark for 30 min and subsequent centrifugation at 3700 rpm for 75 min at 4 °C. Separation of the DNA fragments was obtained in a Megabace 1000 system (GE Health Care). Voltage and time of injection were 3 kV and 80 s. Running was performed at 9 kV for 100 min at 44 °C.

Vibrios were identified using the identification marker *pyrH*, as described previously with minor modifications [21,28,29]. Briefly, PCR was composed of 38.2 µl sterile

MilliQ water, 1.5 µl Mg_2Cl (1.5 mM), 5.0 µl PCR buffer (10 ×), 0.4 µl dNTP's (0.2 mM each), 1.2 µl forward primer (pyrH80F-5′GAT CGT ATG GCT CAA GAA G3′, 20 µM), 1.2 µl reverse primer (pyrH530R-5′TAG GCA TTT TGT GGT CAC G3′, 20 µM), 0.4 µl AmpliTaq DNA Polymerase (2 U/µl), and 2.0 µl template DNA (0.05 µg/µl). The thermal program consisted of (1) 5 min at 95 °C, (2) 3 cycles of 1 min at 95 °C + 2 min 15 s at 55 °C and 1 min 15 s at 72 °C, (3) 30 cycles of 30 s at 95 °C + 1 min 15 s at 55 °C and 1 min 15 s at 72 °C, and (4) a final 7 min at 72 °C. *pyrH* PCR products were purified with the enzyme Exosap, according to the instructions of the manufacturer (GE Health Care). Sequencing was performed as described above using 0.6 µl of each PCR primer (pyrH80F and pyrH530R, 20 µM) and an annealing temperature of 50 °C.

Raw sequence data were transferred to the Gene Builder module within the Kodon package version 2.03 (Applied Maths, Belgium), where consensus sequences were determined. Similarity matrices and phylogenetic trees were constructed in the Mega version 4.0 software [24], using partial 16S rDNA (ca. 500 bp) and *pyrH* (ca. 450 bp) sequences. Trees were drawn using the neighbor-joining method [20] based on Kimura-2P distances with bootstrap analysis using 1000 repetitions. The gene sequence data obtained in this study is also available through the website TAXVIBRIO (http://www.taxvibrio.lncc.br/). The gene sequences were deposited in the GenBank under the accession nos. EU251514-EU251689.

Nitrogen fixation: acetylene reduction test (ARA) and growth in minimal medium

Nineteen representative *Vibrio* isolates were grown six times successively in semisolid NFb medium at 28 °C for 48 h in anaerobic conditions. *Klebsiella* sp. ICB375 and *Escherichia coli* ICB250 were included as positive and negative controls, respectively. N_2 fixation was also evaluated using the acetylene reduction test with the same set of isolates. Cultures were incubated in semisolid NFb medium with 0.6 ml of acetylene in sealed tubes and kept at 28 °C for 48 h. The production of ethylene was quantified by injecting 0.1 ml of the air phase produced in the sealed tubes into a gas chromatographer (Shimadzu GC-14A, equipped with a column PorapaK-N 80/100 – INOX) at 70 °C [30]. The injector and detector were set at 180 and 230 °C, respectively. Each isolate was tested independently by acetylene reduction assay three times.

Results and discussion

The 95 isolates belonged to the *Gammaproteobacteria* according to the partial (ca. 500 bp) 16S rDNA gene sequences (Fig. 1). The majority of the isolates ($n = 76$) fell within the *Vibrio* core group, with highest 16S rDNA gene sequence similarity towards *Vibrio harveyi*, and *V. alginolyticus*. In order to identify these isolates unambiguously, *pyrH* sequences were obtained and compared with a large database [21]. It was shown in a previous study that the *pyrH* gene is a reliable identification marker for vibrios [27–29]. Clearly, most of the *Vibrio* isolates belonged to the species *V. alginolyticus* and *V. harveyi*, according to *pyrH* gene sequences (Fig. 2), although some isolates grouped with the reference strain *V. harveyi* LMG 20370. This latter

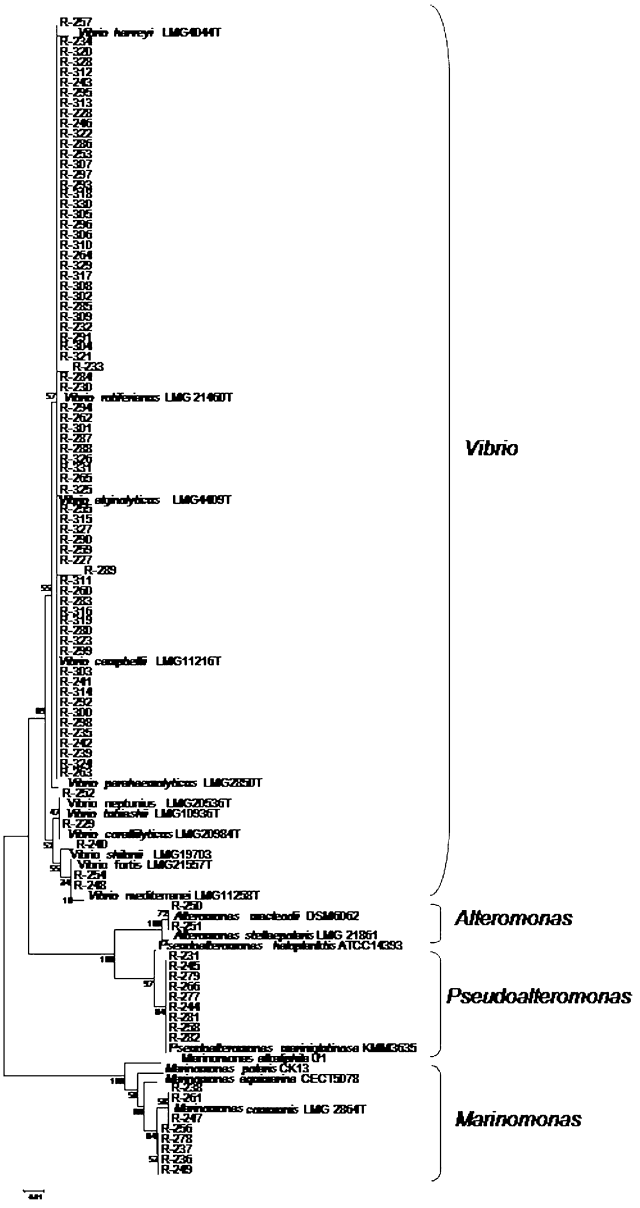

Fig. 1. Phylogenetic tree based on partial 16S rDNA sequences (ca. 500 bp) using the neighbor-joining method and Kimura-2P distances. Bootstrap analysis after 1000 repetitions is shown. Scale bar corresponds to 1% sequence divergence.

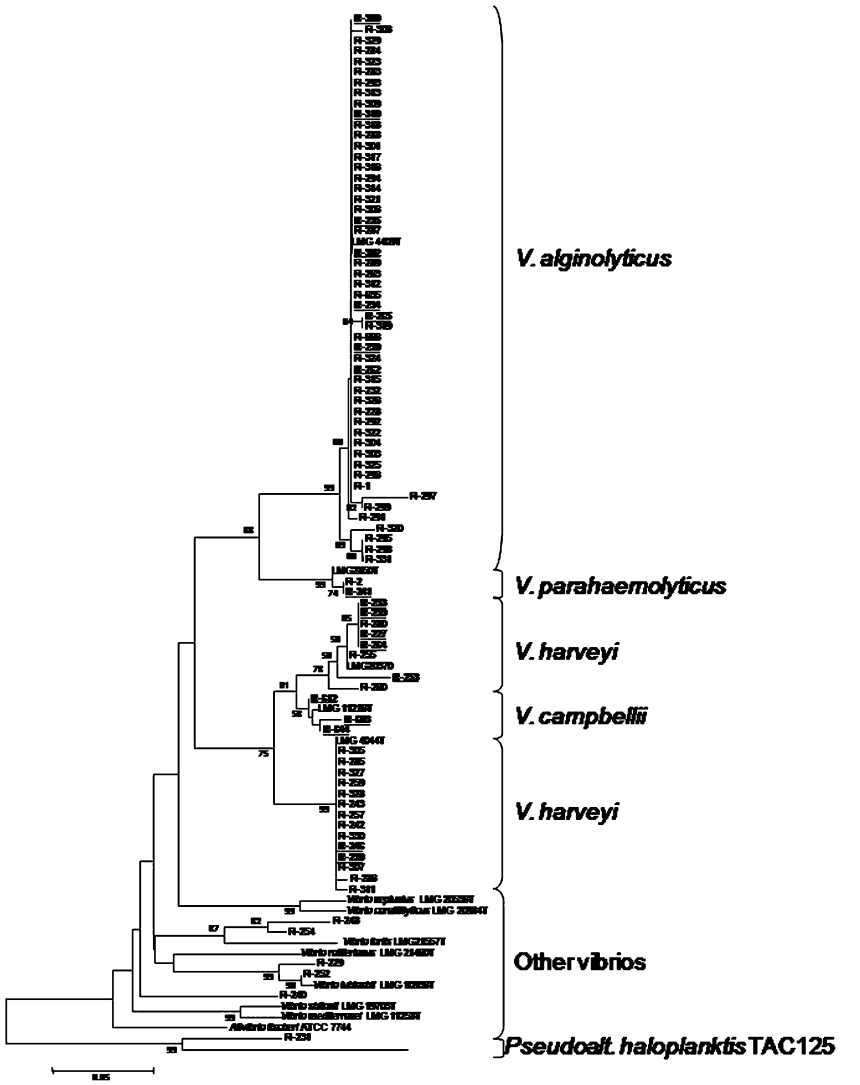

Fig. 2. Phylogenetic tree based on *pyrH* sequences (ca. 450 bp) of vibrios using the neighbor-joining method and Kimura-2P distances. Bootstrap analysis after 1000 repetitions is shown. Scale bar corresponds to 5% sequence divergence. *V. campbellii* R-603, R-612, R-644, and *V. parahaemolyticus* R-2 were isolated from *M. hispida* by using TCBS and were included as controls in this study [28]. Representative strains tested for growth six successive times in NFb and ARA are underlined.

strain formed a separate group by AFLP; the so called AFLP group 31 described by Thompson et al. [25]. The group comprising LMG 20370 possibly represents a new species, but further taxonomic work is needed in order to confirm this hypothesis. Several isolates within the species *V. alginolyticus* and *V. harveyi* were indistinguishable by *pyrH* sequences, suggesting the low taxonomic resolution of this gene for discriminating strains. One single *M. hispida* colony harbored up to eight different colony morphotypes. Overall, the different morphotypes belonged to the same species. Isolates R-288, R-291, R-292, R-293, R-297, R-318, R-324, and R-323 belonged to *V. alginolyticus*, indicating colony variation in this species (Table 1).

All 19 representative isolates (eight *V. alginolyticus*, seven *V. harveyi*, three *V. campbellii* and one *V. parahaemolyticus*) were able to grow six successive times in the nitrogen-free medium, indicating that they indeed fixed N_2 (Table 1; Fig. 2). *V. alginolyticus* isolates showed prolific growth in the nitrogen-free medium. The acetylene reduction test indicated nitrogenase activity in *V. harveyi* R-227, R-230, R-239, R-246, and R-253, the latter of which was an unexpected phenotype for this species. The fact that both *V. alginolyticus* and *V. harveyi* fix N_2 might explain their dominance amongst other species in the mucus of the cnidarians examined in this study. In the holobiont, these vibrios may obtain N_2 via microbial-mediated denitrification within the coral mucus [31].

A single *M. hispida* colony harbored isolates belonging to different species, as was the case for *V. alginolyticus* R-289 and R-310, *Marinomonas* sp. R-261, and *Pseudoalteromonas* sp. R-231. Isolates

allocated to the genus *Alteromonas* were also retrieved. *Alteromonas* sp. R-250 and R-251 appeared in *M. hispida* and *P. caribaeorum*, respectively, and in different locations. *Marinomonas* and *Alteromonas* isolates showed <97% 16S rDNA similarity towards known species. These microorganisms are abundant in the marine environment and are commonly retrieved in culture-dependent diversity studies worldwide, but the isolates obtained in this study differ from currently described species. The isolates associated with the Brazilian cnidarians probably belong to new species yet to be described taxonomically. Work is under way to describe the new taxa and to understand better their role in coral health. With the exception of the new *Marinomonas* isolates that showed some sort of host specificity for *M. hispida*, all other bacterial species appeared in multiple cnidarian hosts, indicating that these sympatric cnidarians share their N_2-fixing microbiota.

One of the most notorious groups of coral-associated bacteria is the *Vibrio* core group, which are pathogens [1,3,8,12,17]. We show here that several *Vibrio* isolates are able to fix N_2 and in doing so they might be considered coral mutualists. It is well known that vibrios of the core group may respond swiftly to changes in environmental conditions. For instance, the doubling time in vibrios of the core group may be below 15 min, particularly at temperatures higher than 25 °C and in carbon-rich environments such us the coral mucus [22]. The results presented in this study suggest that *V. alginolyticus*, *V. harveyi*, *V. campbellii*, and *V. parahaemolyticus* isolates might have a positive effect on coral health by fixing N_2, but further work in aquaria is needed to confirm if the same strains can both fix N_2 and cause infection in corals. It might be that under stressful conditions such as high sea water temperature and high nutrient loads (i.e. high concentrations of dissolved ammonia, phosphate, and organic matter) these vibrios will act as opportunistic pathogens, outcompeting other species present in the coral mucus [10]. Our taxonomic study suggests that N_2 fixation is a common phenotypic characteristic among different *Vibrio* species, including putative coral pathogenic species. Work is currently under way to unravel the genomic properties of representative N_2-fixing isolates obtained in this study.

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