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

Luciane A. Chimetto[a], Marcelo Brocchi[a], Cristiane C. Thompson[b], Roberta C.R. Martins[c], Heloiza R. Ramos[c], Fabiano L. Thompson[d,*]

[a]*Departamento de Microbiologia e Imunologia, Instituto de Biologia, Universidade de Campinas (UNICAMP), Brazil*
[b]*Instituto Oswaldo Cruz, Rio de Janeiro, Brazil*
[c]*Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo (USP), Brazil*
[d]*Department of Genetics, Institute of Biology, Federal University of Rio de Janeiro (UFRJ), Brazil*

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

*Corresponding author at: Av. Brigadeiro Trompowsky, s/no. Centro de Ciências da Saúde, Depto. de Genética, Bloco A, Sala 105-A2, Ilha do Fundão – Rio de Janeiro – RJ – CEP: 21941-590, Brazil. Tel.: +55 21 25626382; fax: +55 21 25626396.
E-mail address: Fabiano.Thompson@biologia.ufrj.br (F.L. Thompson).

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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