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RESEARCH NOTE

Culturable bacteria associated with the mucus of the zoanthid *Palythoa caribaeorum* (Cnidaria, Anthozoa) from Northeast of Brazil

Bacterias cultivables asociadas al mucus del zoantídeo *Palythoa caribaeorum* (Cnidaria, Anthozoa) en el noreste de Brasil

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Abstract.- The zoanthid *Palythoa caribaeorum* secretes a large quantity of mucus that acts as a substrate for microbial communities. Culturable bacteria associated with *P. caribaeorum* mucus in northeastern Brazil were evaluated through 16S rRNA gene sequences. Proteobacteria was the dominant group, followed by Actinobacteria and Firmicutes. *Vibrio* was the most common genus, although other groups with the ability to produce biosurfactants and compounds with antimicrobial activity were also present. The studies of culturable marine bacteria may contribute to the understanding of the associated microbial community, opening new opportunities to explore biotechnological potential of microbiota.

Key words: Coral reefs, zoanthid, mucus, microbial community, genome sequencing

INTRODUCTION

Zoanthids are cnidarians phylogenetically close to corals and abundant in reef ecosystems of tropical shallow waters, although deep-water species have also been reported (Reimer *et al*. 2007). In Brazil, zoanthids are an important component of the benthic fauna and are distributed along the littoral coast. The zoanthid *Palythoa caribaeorum* Duchassaing & Michelotti, 1860 occurs in the western Atlantic and is frequently found in Brazilian reefs forming large carpets due to its high growth rate (Silva *et al*. 2015).

Colonies of *P. caribaeorum* secrete a large quantity of mucus (Campos *et al.* 2015). The mucus produced by these and other Cnidarians is composed of a glycoprotein and lipid matrix (Bythell & Wild 2011). It performs various functions including cleaning sediment of colonies, defense against environmental stresses such as high temperatures and UV radiation, and as substrate for the growth of microorganisms (Glasl *et al*. 2016). Many bacteria associated with the tissues of marine organisms are able to form biofilms that offer benefits to the hosts such as nitrogen fixation, nutrition and animal protection.

The relationship between bacteria, coral colonies and zoanthids has been investigated in recent years (*e.g*., Sun *et al*. 2014, Bourne *et al*. 2016). *P. caribaeorum* bacterial diversity seems to have a relevant role in the maintenance of colony health, and a biotechnological potential still poorly explored (Carlos *et al*. 2013, Paulino *et al*. 2017). For instance, nitrogenase activity has been found to indicate the presence of bacterial groups with nitrogen fixation abilities (Chimetto *et al*. 2008), oil-derived hydrocarbon degradation (Campos *et al*. 2015), production of bioactive compounds with anti-microbial activity (Pereira *et al*. 2017), and hemolytic activity similar to palytoxin, suggesting colony defense (Seemann *et al*. 2009).

Therefore, the objective of the present study was to evaluate the bacterial diversity associated with the mucus of *P. caribaeorum* from Northeastern Brazil, through the sequencing of the 16S rRNA gene. This identification of the taxonomic composition of culturable bacterial communities may help to uncover their biological potential.

Materials and methods

Collection location and conditions

Data collection was performed during low tide in Porto de Galinhas reefs (8º30'20''S; 35º00'34''W), Ipojuca, Pernambuco, Brazil. Four collection expeditions were performed during 2016, in the months of April, May, June and October.

Mucus from *P. caribaeorum* colonies (20 in each collection) (Fig. 1) was collected in 50 mL sterile centrifuge tubes by lightly scraping the emerged colonies. Samples were packed in Styrofoam boxes filled with ice and immediately transported to the laboratory, where the microorganisms were isolated and purified.

Isolation and preservation of strains

Isolation of heterotrophic bacteria from *P. caribaeorum* mucus was performed using marine agar (Difco®), based on surface and pour-plate techniques. A volume of 3 mL of each mucus sample was collected and inoculated on Petri dishes containing the medium. Plates were incubated at 30 ºC for 3 to 6 days. Representative morphotypes obtained were sub-cultured and purified to exhaustion obtaining pure bacterial cultures. All isolates were analyzed by the Gram coloration technique. Isolates were resuspended in marine agar with 15% glycerol and frozen at -80 °C.

DNA extraction and amplification of the DNA obtained

DNA was extracted by thermal shocking. A small quantity of each isolated colony was collected with an inoculation loop, transferred to 2 mL tubes and dissolved in 100 µL of sterilized ultrapure water. The material was mixed in a vortex and brought to 98 ºC for 10 min. A thermal shock in a freezer at -20 ºC for 10 min was performed, followed by centrifugation at 14,100 × g for 20 min. The supernatant was transferred to sterilized tubes.

After quantification using Qubit Kit (Invitrogen), the quality was confirmed by 1% agarose gel electrophoresis, 25 ng of DNA from each sample was employed for the partial 16S rRNA gene amplification by using the primers 27F (5'AGA GTT TGA TCM TGG CTC AG) and 1492R (5'TAC GCY TAC CTT GTT ACG ACT T). The 50 µL reactions included 5 µL of DNA template, 5 µL (20 pmol μL^{1}) of each primer (forward and reverse), 5 µL of dNTP's, 5 µL of PCR buffer, 0.1 units of Taq DNA polymerase (Fermentas®) and 22 µL of ultrapure water. The reaction cycles were: 94 ºC (5 min), 30 cycles at 94 ºC (1 min), 62 ºC (1 min) and 72 ºC (3 min), followed by an extension step at 72 ºC (10 min). Amplification success was verified by electrophoresis in 1% agarose gel, stained with GelRed at a 1:10,000 dilution and the products were purified using QIAquick PCR Purification kit (QIAGEN®). Purified products were quantified in a spectrophotometer Thermo Fisher Scientific® and diluted to a final concentration of 100 ng μL^{-1}, for the sequencing reactions.

Figure 1. Exposed colonies of *Palythoa caribaeorum* (red arrow) growing on the Porto de Galinhas reefs, Pernambuco, Brazil (Photo: Liany Melo) / Colonias expuestas de *Palythoa caribaeorum* (flecha roja) creciendo en los arrecifes de Porto de Galinhas, Pernambuco, Brasil (Foto: Liany Melo)

Sequencing of 16S rRNA

Sequencing reactions were performed using DYEnamic ET Dye Terminator kit (MegaBACE®). Reactions were conducted in a volume of 10 µL containing: 100 ng of PCR product, 2.0 µL of DYEnamic, 25X PCR buffer (20 mM Tris-HCl pH= 8.4), 25 pmoles of oligonucleotide forward and water (ultrapure q.s.p. 20 µL). The products were purified with 75% isopropanol, washed with 70% ethanol, resuspended in 10 µL of formamide and taken for sequencing in ABI 3100 - Applied Biosystems®, in a capillary system. Sequences were analyzed by nucleotide similarity in GeneBank data base, accessed through the National Center for Biotechnology Information and the algorithm BLASTn.

Phylogenetic analysis

ClustalW was employed for sequence alignment. Similarity matrices and phylogenetic trees were constructed by Molecular Evolutionary Genetics Analysis (MEGA 7.0). Evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length of 969.30764129 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. These evolutionary distances were computed using the number of differences method (Nei & Kumar 2000) and are in the units of the number of base differences per sequence. The robustness of each topology was checked by 1,000 bootstrap replications. Gene sequences of the identified bacteria were obtained from BLASTn search in GenBank with the highest sequence similarities against reference strains. GenBank accession numbers of the 16S rRNA gene sequences (MW281423-MW281459) are provided for this study.

Results and discussion

A total of 37 isolates were recovered from *P. caribaeorum* colony mucus, sampled from the reefs of Porto de Galinhas. Analysis of the sequences' homology among the isolates and reference strains, performed by phylogenetic emphasis, revealed that Proteobacteria was the predominant bacterial phylum associated with *P. caribaeorum* mucus (Fig. 2). Gammaproteobacteria including *Alcanivorax*, *Vibrio*, *Pseudomonas*, *Pseudoalteromonas* and *Photobacterium* represented 73.68% of the isolates, followed by Alphaproteobacteria (*Labrenzia*, *Pseudovibrio*, *Altererythrobacter* and *Paracoccus*) with 13.15%. Indeed, these Gram-negative bacteria groups are amply distributed in the world's oceans. On the other hand, Gram-positive bacteria were less frequent. Actinobacteria (*Micrococcus*, *Nesterenkonia* and *Brevibacterium*) represented 10.52% and Firmicutes (*Bhargavaea*) 2.83%. The dominance of Proteobacteria and its high generic diversity found here has been reported in corals and other marine invertebrates (Bourne *et al*. 2016), suggesting the importance of these bacteria to host organisms in terms of mutualistic existence. Similar results were observed in studies with zoanthids, where Proteobacteria dominance was common (Carlos *et al*. 2013, Sun *et al*. 2014, Paulino *et al*. 2017, Pereira *et al*. 2017). Gammaproteobacteria were also dominant in the sample of *P. caribaeorum* mucus collected from São Sebastião Channel, São Paulo, Brazil (Carlos *et al*. 2013), whereas Alphaproteobacteria dominated the isolates associated with *Palythoa australiae* collected from the South China Sea (Sun *et al*. 2014). In the reefs of Carapibus, in the neighbor state of Paraíba, Pereira *et al*. (2021) observed a dominance of Firmicutes, especially *Bacillus*, while Gammaproteobacteria represented only 16% of the isolates, when analyzing antimicrobial activity of bacteria associated with *P. caribaeorum*.

Results indicate that the dominance of Gammaproteobacteria isolates in *P. caribaeorum* mucus may be influenced by sewage effluents due to the exacerbated urbanization in the vicinity of Porto de Galinhas reefs. Paulino *et al*. (2017) observed a significant difference in bacterial diversity associated with *P. caribaeorum* colonies between two collection localities on the coast of Alagoas, Brazil. Alphaproteobacteria were dominant in reefs suffering from less anthropogenic influence. In turn, the most impacted and exposed reefs to urban waste presented a dominance of Gammproteobacteria and included a high proportion of groups that cause human diseases such as, *Pseudomonas*, a genus also found in this study. Local anthropogenic impacts on coral mucus bacterial communities could also be represented as an increase of bacterial species related to cnidarian diseases (Hussein *et al*. 2022).

Despite the relatively high generic diversity (13 genera), most taxa had few isolates. Only *Vibrio* showed a representative number of isolates. The dominance of *Vibrio* (52.63% of total isolates), agrees with previous studies (Chimetto *et al*. 2008, 2009). A total of 14 isolates (samples 2, 5, 12, 14, 18, 29, 38, 40, 50, 51, 55, 58, 73, 76) identified as *Vibrio* spp. showed low sequence similarity to known species, suggesting that these may be new taxa. Sample 65 presented homology with *V. communis*, whereas samples 39, 61, 64, 66 were homologous with *V. harveyi*. Vibrios are commonly associated with coral infections and diseases and may significantly increase their dominance before and during bleaching events. However, Vibrios are also common in healthy colonies (Chimetto *et al*. 2008, 2009).

Figure 2. Evolutionary relationships of taxa inferred by partial 16S rRNA gene sequences (650 bp). The percentage of replicate trees and bootstrap test (1000 replicates), is shown next to the branches. All positions with less than 95% coverage were removed. The analysis involved 88 nucleotide sequences. There was a total of 369 positions in the final dataset. Strains isolated in this study are highlighted in red / Relaciones evolutivas de taxones inferidas por secuencias parciales del gen 16S rRNA (650 pb). El porcentaje de árboles replicados y prueba de bootstrap (1000 repeticiones) se muestra junto a las ramas. Se eliminaron todas las posiciones con menos del 95% de cobertura. El análisis involucró 88 secuencias de nucleótidos. Hubo un total de 369 posiciones en el conjunto de datos final. Las cepas aisladas en este estudio se destacan en rojo

The presence of strains related to the Harveyi clade (*V. harveyi*, *V. parahaemolyticus* and *V. alginolyticus*) may suggest the occurrence of nitrogen fixation processes in this host (Chimetto *et al*. 2008). Nitrogen fixation by *Vibrio* spp. comprises part of the mutualistic relationship between these bacteria and corals. Bacteria can obtain nitrogen through denitrification provided by other species associated with host mucus. For example, *Pseudovibrio* spp. are known for dominating microbial communities associated with diverse marine invertebrates and some of their strains are capable of carrying out denitrification. *Pseudovibrio dentirificans* participates in denitrification processes, *i.e*., reduction of nitrate or nitrite into gaseous products such as nitrogen gas (Shich *et al*. 2004), and could be involved in the chain of nitrogen fixation by Vibrios.

Pseudoalteromonas, *Pseudovibrio*, *Alcanivorax* (Rizzo *et al*. 2014), *Vibrio* (Graziano *et al*. 2016), and *Labrenzia* (Gao *et al*. 2019) identified in this study have been reported to produce biosurfactants. These compounds are capable of removing hydrocarbons in contaminated environments. For instance, *Labrenzia* species are known for degrading aromatic compounds. *Alcanivorax* is a biological marker for pollution in seawater and is generally a dominant group in environments with oil derivatives as a carbon source (Zadjelovic *et al*. 2020). In oil spill bioremediation processes, the introduction of nutrients, such as phosphorous and nitrogen, induce an increase in these bacteria populations that degrade hydrocarbons. Pereira *et al*. (2017) verified that *Alcanivorax* strains were present in *P. caribaeorum* mucus, but not in the water or surrounding marine sediments, reinforcing the association of these bacteria with their hosts.

Bacterial taxa with known antimicrobial activities were reported in this study, such as *Photobacterium* (Oku *et al*. 2008), *Micrococcus* (Kuang *et al*. 2015), *Labrenzia* (Sharma *et al*. 2019), *Paracoccus* (El Samak *et al*. 2018), and *Vibrio* (Pereira *et al*. 2021). Bacterial isolates with antibacterial activity may act as first line of defense to protect the coral host against pathogens (Hussein *et al*. 2022). Additionally, *Brevibacterium* and *Vibrio* may be involved in mucus toxicity. Seeman *et al*. (2009) reported that *Bacillus* and *Brevibacterium* strains showed haemolytic activity in *P. caribaeorum* mucus, suggesting that these bacteria may be involved in palytoxin synthesis. Palytoxin is a potent and dangerous toxin produced by several marine species and was firstly detected in zoanthids of the genera *Palythoa*, *Protopalythoa* and *Zoanthus* (Gleibs & Mebs 1999). Likewise, *Vibrio* species have been suggested to be involved in tetrodotoxin synthesis, by the occurrence of this potent neurotoxin in phylogenetically distinct organisms (Wang *et al*. 2008).

Although a reduced number of isolates were evaluated in this study, the diversity found here is similar to the bacterial community associated with *P. caribaeorum* mucus from São Sebastião Channel, Brazil (Carlos *et al*. 2013, Pereira *et al*. 2017). These authors reported that the bacterial community associated with this host was stable and minimally influenced by temperature when compared to surrounding marine water and sediment communities and suggested that this stability was due to antimicrobial properties. Bacterial communities with the dominance of *Proteobacteria* in the coral mucus of Brazilian samples reveal that these bacteria are important in protecting cnidarian health, as well as other marine invertebrates around the world (Lo Giudice & Rizzo 2022).

Studies of culturable marine bacteria may contribute to understand the microbial community associated with the hosts, opening new opportunities to explore biotechnological potential of different bacterial groups. Furthermore, it is relevant to understand the microbes associated with marine animals in order to create health parameters, since significant changes in associated bacterial diversity may indicate environmental stress.

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