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Alcanivorax dieselolei, an alkane-degrading bacterium associated with the mucus of the zoanthid *Palythoa caribaeorum* (Cnidaria, Anthozoa)

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(With 2 Figures)

Abstract

Analyses of 16S rDNA genes were used to identify the microbiota isolated from the mucus of the zoanthid *Palythoa caribaeorum* at Porto de Galinhas on the coast of Pernambuco State, Brazil. This study is important as the first report of this association, because of the potential biotechnological applications of the bacterium *Alcanivorax dieselolei,* and as evidence for the presence of a hydrocarbon degrading bacterium in a reef ecosystem such as Porto de Galinhas.

Keywords: 16S rDNA, microbiota, biodegradation, hydrocarbons, cnidarians.

Alcanivorax dieselolei, uma bactéria degradadora de alcanos associada ao muco do zoantídeo *Palythoa caribaeorum* (Cnidaria, Anthozoa)

Resumo

Análises dos genes 16S rDNA foram empregadas para identificar a microbiota isolada do muco do zoantídeo *Palythoa caribaeorum* de Porto de Galinhas, litoral do estado de Pernambuco, Brasil. Este estudo é importante pelo ineditismo dessa associação, pelas relevantes aplicações biotecnológicas da bactéria *Alcanivorax dieselolei* e pela indicação da presença de uma bactéria degradadora de hidrocarbonetos em um ecossistema recifal como o de Porto de Galinhas.

Palvras-chave: 16S rDNA, microbiota, biodegradação, hidrocarbonetos, cnidários.

1. Introduction

Coral reefs are complex ecosystems that provide microniches for enormous diversities of microorganisms (Ainsworth et al., 2010) associated with marine invertebrates such as sponges, cnidarians, and mollusks (Sfanos et al., 2005). Cnidarians, especially corals, have many microorganisms associated with their tissues and mucus (Rohwer et al., 2002; Chimetto et al., 2009; Castro et al., 2010). Coral mucus – a micro-layer of polysaccharides and glycoproteins deposited over the surfaces of these animals – has been found to contain representatives of the three primary domains Archaea, Eubacteria and Eukarya (Meikle et al., 1988; Wegley et al., 2007).

While a number of workers studies have examined the microbiota associated with corals and other marine invertebrates (Castro et al., 2010; Menezes et al., 2010; Trindade-Silva et al., 2012), zoanthids have been little examined in that respect, although some investigations of the diversity of bacteria found on zoanthids by Chimetto et al. (2008, 2009, 2011) identified 16S rDNA sequences of bacteria of the genus *Vibrio*.

The zoanthid *Palythoa caribaeorum* (Duchassaing and Michelotti, 1860) is a cnidarian commonly found on reefs in the Caribbean region and Brazil (Mueller and Haywick, 1995). It can form dense layers on reefs as it is a strong competitor for space (Pérez et al., 2005), is tolerant of environmental stresses (Sebens, 1982), demonstrates a high reproductive capacity (Acosta and Asbahr, 2000), and liberates a potent non-protein toxin called palytoxin (Seemann et al., 2009).This cnidarian is popularly known as "baba-de-boi" ("cattle spittle") as it secretes a very viscous mucus over the surface of the colony during low tides that can shelter other marine microorganisms (Ainsworth et al., 2010).

The bacterium *Alcanivorax dieselolei* was originally isolated from seawater samples and marine sediments contaminated by oil in the Pacific Ocean and described by Liu and Shao (2005). These bacteria, as well as other

species of the same genus, predominate in temperate marine environments impacted by oil (Cappello et al., 2007).

The present work represents the first record of *A. dieselolei*, a specie capable of degrading petroleum derivatives, in the Atlantic Ocean and associated with the zoanthid *P. caribaeorum*. The potential biotechnological applications of the bacterium are discussed.

2. Material and Methods

Mucus was collected from exposed reef colonies of *P. caribaeorum* (Figure 1) during low tide periods in May/2010 at Porto de Galinhas on the southern coast of Pernambuco State, Brazil (8°30'24''S; 34°59'52''W) (Figure 2). Mucus samples were scraped and placed in sterile 50 mL centrifuge tubes. Heterotrophic bacteria were isolated from the mucus by inoculating 2 mL of the collected mucus onto Marine Agar 2216 (Difco®) using the pour plate technique and incubating the cultures at 30 °C for five days.

DNA was extracted from individual bacterial colonies using the thermal shock technique, in which a small quantity of material from each colony was collected and resuspended in 100 µl of ultrapure sterilized water, exposed to temperatures of 98 °C for 10 min. and –20 °C for 10 min. and subsequently centrifuged; the supernatants were transferred to sterile tubes.

Figure 1. Colony of zoanthid *Palythoa caribaeorum* (Photo: Liany Melo).

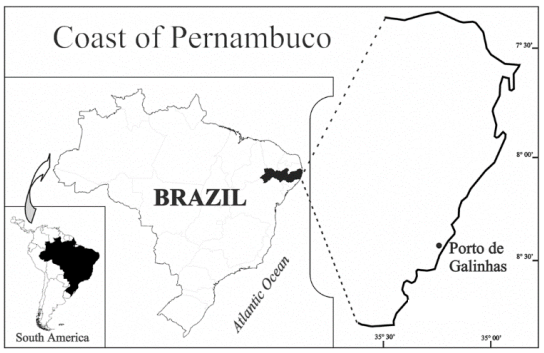

Figure 2. Map of the coast of the state of Pernambuco indicating the collection point.

The total DNA from each sample was used as a template for amplifying the 16S rDNA segments of Eubacteria using the primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' TAC GCY TAC CTT GTT ACG ACT T 3') (Rohwer et al., 2002). The PCR reactions were performed in a final volume of 50 µl that included: 50 ng of the template DNA, 10 pmol of each primer, 200 µM dNTPs, 5 µl PCR buffer, 5 U of Taq polymerase for DNA (Fermentas®), and 22 µl of ultrapure sterilized water. The thermocycler program consisted of: (1) 5 minutes at 94 °C; (2) 30 cycles of 1 minute at 94 °C, 1 minute at 62 °C, and 3 minutes at 72 °C, and; (3) 10 minutes at 72 °C. The PCR products were purified using the QIAquick PCR Purification kit (QIAGEN®).

The sequencing reactions were performed using BigDye® (ABI) and DYEnamic ET (Amersham Biosciences®) chemistry for the ABI Prism (Model 3100). The reactions were performed in a final volume of 10 µl containing: 100 ng of template DNA (the amplification products), 2 µl of marked nucleotides, 25X PCR buffer (20 mM Tris-HCl pH=8.4), 25 pmoles of each primer (forward and reverse), and previously sterilized Milli-Q water (q.s.p. 20 µl). These reactions were performed using the same program described above for the PCR reactions.

The qualities of the resulting DNA sequences were evaluated using Sequencing Analysis 3.4 software (Applied Biosystems®, Foster City, CA, EUA). The sequences were submitted to nucleotide similarity consultations of the data available at GenBank, on the the NCBI (National Center for Biotechnology Information) web site using the *BLASTn* algorithm (Altschul et al., 1990).

3. Results and Discussion

Fifty bacterial isolates were obtained from the mucus of *P. caribaeorum* collected on reefs in Porto de Galinhas, Pernambuco State, Brazil. The dominant group among the isolated bacteria was γ-Proteobacteria, with 36 isolates (72%), followed by α-Proteobacteria and Actinobacteria with six isolates each (12%), and Firmicutes with two isolates (4%); one isolate of *A. dieselolei* was encountered among the γ-Proteobacteria. Comparisons of the nucleotide sequence of the 16S rDNA gene of the bacterium isolated from *P. caribaeorum* mucus with sequences deposited in the GenBank indicated similarities 100% with the sequences of the group that performed the complete genome sequence of *A. dieselolei* Type Strain B5 (Lai et al., 2012). The nucleotide sequence was deposited in GenBank with the accession number KF545933.

We therefore report here the first known association of the bacteria *A. dieselolei* with the zoanthid *P. caribaeorum.* Sequences of this bacteria had only previously been obtained from seawater and marine sediment samples (Liu and Shao, 2005; Tapilatu et al., 2010), as is true for most of the other isolates and 16S rRNA gene sequences of other bacterial strains of the genus *Alcanivorax* (Liu and Shao, 2005; Cappello et al., 2007; Rivas et al., 2007; Wu et al., 2009). Some presently non-identified strains of the genus

Alcanivorax have been isolated from marine invertebrates such as sponges and gorgonians (Sfanos et al., 2005), but our current report is the first record of any association between *A. dieselolei* and *P. caribaeorum,* and its first reported occurrence in the Atlantic Ocean.

Among the hydrocarbonoclastic bacteria, the genus *Alcanivorax* comprises Gram-negative, aerobic, and halophytic species that have the capacity to metabolize alkanes hydrocarbons as a carbon source and for energy and have been used in bioremediation projects in polluted marine environments (Yakimov et al., 1998; Liu and Shao, 2005). The genus *Alcanivorax* comprises six described species: *Alcanivorax borkumensis* (Yakimov et al., 1998), *Alcanivorax venustensis, Alcanivorax jadensis* (Fernández-Martínez, 2003), *Alcanivorax dieselolei* (Liu and Shao, 2005), *Alcanivorax balearicus* (Rivas et al., 2007), and *Alcanivorax hongdengensis* (Wu et al., 2009).

The fact that *A. dieselolei* was isolated from colonies of *P. caribaeorum* is quite intriguing because, while the beach at Porto de Galinhas has been heavily visited by tourists, it does not appear to be contaminated to any significant degree by petroleum compounds in spite of its proximity to the shipping port at Suape. On the other hand, large numbers of swimmers and divers do visit this beach and use solar protection lotions containing hydrocarbons such as benzophenone, oxybenzone, or parabens. These compounds may serve as energy and carbon sources for bacteria that metabolize only alkanes and reject other carbon resources such as sugars and amino acids (Lorenzo, 2006).

The presence of the bacteria *A. dieselolei* in *P. caribaeorum* mucus might also be at least partially explained by the presence of high concentrations of phosphorus and nitrogen compounds (commonly used in bioremediation projects to break bacterial dormancy) in that milieu (Cappello et al., 2007). It is also known that bacteria of the genus *Vibrio*, which can fix nitrogen, are associated with this same zoanthid (Chimetto et al., 2008) so that *A. dieselolei* may be using these compounds as energy sources as these bacteria show denitrification activity and *Alcanivorax* strains are known to contribute to the degradation of nitrogen and phosphorus compounds that accumulate in organically enriched coastal areas (Nakano et al., 2009).

Another possible explanation for the presence of this bacterium in association with *P. caribaeorum* would be the natural production of aliphatic compounds by that animal. According to Yakimov et al. (2007), the isolation of the bacteria *A. borkumensis* from marine invertebrates may reflect the existence of specific ecological niches containing hydrocarbons produced by those animals themselves. The presence of *A. borkumensis* in non-polluted environments (Kasai et al., 2001) indicates that *A. dieselolei* might likewise naturally exist in environments not polluted by oil. It will be necessary to monitor the Porto de Galinhas beach area for possible environmental impacts in any case, as these bacteria can be used as bioindicators of water contamination by either high or low levels of long-chain hydrocarbons (Fernández-Martínez et al., 2003).

Other genera (similarity indices ≥ 97%) with bacterial species with known capabilities to degrade alkanes derived from petroleum were also found associated with the secreted mucus of *P. caribaeorum* in the present work, including *Altererytrobacter, Pseudomonas, Rhodococcus*, and *Stappia* – which is interesting from the point of view of possible biotechnological applications. The present study also represents the first report of the association of these bacteria with the zoanthid *P. caribaeorum,* a cnidarian that has been only poorly examined in terms of its associated microbiota in spite of its abundance in most reef environments in the western Atlantic.

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