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Diversity and pathogenic potential of vibrios isolated from Abrolhos Bank corals

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Summary

We performed the first taxonomic characterization of vibrios and other culturable microbiota from apparently healthy and diseased Brazilian-endemic corals at the Abrolhos reef bank. The diseases affecting corals were tissue necrosis in *Phyllogorgia dillatata*, white plague and bleaching in *Mussismilia braziliensis* and bleaching in *Mussismilia hispida*. Bacterial isolates were obtained from mucus of 22 coral specimens originated from the Abrolhos Bank (i.e. Itacolomis reef, Recife de Fora reef and Santa Barbara Island) in 2007. Vibrios counts in the water and coral mucus were approximately 104 cfu ml^{-1} and 106 cfu ml^{-1} respectively. One hundred and thirty-one representative vibrio isolates were identified. Most vibrio isolates ($n = 79$) fell into the core group using the *pyrH* identification marker. According to our analysis, diseased corals did not possess a unique vibrio microbiota. Vibrio species encompassed strains originated from both apparently healthy and diseased corals. The pathogenic potential of representative vibrio isolates (*V. alginolyticus* 40B, *V. harveyi*-like 1DA3 and *V. coralliilyticus* 2DA3) were evaluated in a standardized bioassay using the animal model *Drosophila melanogaster* and caused 25–88% mortality. This is the first taxonomic characterization of the culturable microbiota from the Brazilian-endemic corals. Endemic Brazilian corals are a reservoir of the vibrio core group. *Vibrio alginolyticus*, *V. harveyi* and *V. coralliilyticus* are dominant in the mucus of these corals and may be a normal component of the holobiont.

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Brazilian coralline reefs are considered as a priority area for biodiversity conservation in the Atlantic due to their small size (5% of Atlantic reefs) and high endemism levels (up to 50% in reef corals) in a highly threatened coastline (Moura, 2002; Moura and Francini-Filho, 2002). The Abrolhos Bank is a 42 000 km^2 enlargement of the continental shelf of Bahia and Espirito Santo states, eastern Brazil, representing the largest and richest reef complex in the South Atlantic (Leão and Dominguez, 2000). Coral reefs have been formed in the last 5000 years in this area, and distributed in a range of cross-shelf strata. Coastal shallow reefs are subjected to high fishing pressure and sediment loads, whereas mid-shelf deeper reefs are less impacted (Francini-Filho and Moura, 2008a). Main reef builders belong to genus *Mussismilia*, an endemic group of massive scleratinian corals (Leão and Ginsburg, 1997; Nunes *et al*., 2008). The coral Mussismilia appears to be threatened by extinction (Francini-Filho *et al*., 2008). *Mussismilia braziliensis* and *M. hispida* represent over 70% of the reef framework mass in some areas, this latter species having a wide geographical distribution (from Maranhão to Santa Catarina states) and the former restricted to the Bahia state. The Abrolhos Bank sustains a great diversity of marine life, potentially playing a major role in the homeostasis of the whole South Atlantic Ocean as a key productivity and nursery area (Dutra *et al*., 2006). The region hosts important reef fisheries targeting snappers (family *Lutjanidae*), groupers (family *Serranidae*) and other valuable coastal resources (e.g. shellfish), with yields of up to 1000 tones caught annually (Francini-Filho and Moura, 2008b). Overfishing has been deemed as one of the main causes of reef degradation since herbivorous

fish play a key role in the control of algae and dissolved organic nutrient cycling (Roberts, 1995). Higher algae cover might favour the proliferation of potentially pathogenic bacteria, e.g. vibrios, by means of dissolved organic matter production (Dinsdale *et al*., 2008).

Diseases affecting these corals have been frequently observed in the last 5 years. Local stressors (e.g. fishing, sediment resuspension and discharge of high nutrient loads from coastal sources) (Segal *et al*., 2008) and global warming are hypothesized as the main drivers of the disruption of the coral holobiont homeostasis. The concept of the coral holobiont put forward by Rohwer and colleagues (2002) suggests a close interdependence and co-evolution between the coral host, its symbiont zooxanthellae and associated microbiota (Rosenberg *et al*., 2007; Weiss and Allemand, 2009). This concept considers that microbes and zooxanthellae play crucial roles within the holobiont, providing the host with nutrients and protection. Beneficial roles played by the coral microbiota include (i) antimicrobial activity against pathogens, (ii) immunoestimulant effects and (iii) nutrition of the host. Cyanobacteria, vibrios and alphaproteobacteria appear to be the main players in nitrogen fixation within the coral mucus (Lesser *et al*., 2004; Chimetto *et al*., 2008; Olson *et al*., 2009; Reis *et al*., 2009).

Global warming and pollution would induce a proliferation of potentially pathogenic vibrio strains. There is mounting evidence that vibrios are the main players in the health of corals worldwide (Raina *et al*., 2009; Vega-Thurber *et al*., 2009). Vibrios tend to dominate the microbiota of environmental settings with high loads of nutrients and at high temperature (Eilers *et al*., 2000). The landmark studies of Rosenberg and co-workers in the middle 1990s suggested that bacteria could be the main cause of coral death, particularly during bleaching (Rosenberg *et al*., 2007). Subsequent studies proved that *Vibrio shilloni* was the aetiological agent of bleaching in *Oculina patagonica*. Temperature is the trigger for the expression of virulence factors by this vibrio during bleaching in *O. patagonica*. *Vibrio coralliilyticus* is also an important coral pathogen. A zinc-metalloprotease produced by *V. coralliilyticus* P1 caused photoinhibition of zooxanthellae and host tissue lysis at high seawater temperature (Sussman *et al*., 2009). So far no data are available on the diversity of the culturable microbiota (vibrios) of *Mussismilia hispida*, *M. braziliensis* and *Phyllogorgia dilatata* of the Abrolhos Bank (Castro and Pires, 2001). This type of data would be useful to better understand these holobionts. In the present study, we performed the first taxonomic characterization of the culturable microbiota of these coral species, comprising vibrios and other heterotrophic bacteria. In order to gain a better understanding of the cnidarian microbiota diversity involved with infections, we sampled apparently healthy and diseased coral specimens. Apparently healthy corals had normal tissue and colour determined visually. The diseased *P. dilatata* from Recife de Fora Reef suffered of tissue necrosis with clear exposure of the skeleton. The diseased *M. braziliensis* from Santa Barbara Island showed clear signs of white plague (Francini-Filho *et al*., 2008), whereas the diseased *M. braziliensis* and *M. hispida* from Itacolomis reef were bleached. In addition, we evaluated the pathogenicity of representative vibrio isolates. We used the animal model *Drosophila melanogaster* as a proxy for screening potential virulence in vibrios (details in Appendix S1).

Total heterotrophic bacteria and vibrios counts in the seawater were approximately 10^4 cfu ml^{-1}. Around 70% of the bacteria growing on Marine Agar belonged to vibrios as revealed by bacterial growth on TCBS plates and further molecular identification (Figs S1–S5). Vibrios counts in the *M. braziliensis* and *M. hispida* mucus were, respectively, 4.9×10^6 cfu ml^{-1} ($\pm$ 2.4×10^6) and 4.4×10^6 cfu ml^{-1} ($\pm$ 1.3×10^6), whereas vibrios counts in the seawater were 1.0×10^4 cfu ml^{-1} ($\pm$ 1.0×10^3). A clear difference was observed in the coral mucus and seawater vibrio counts, indicating that corals are a reservoir of vibrios. Other studies have shown that coral mucus harbour a high number of vibrios. Diseased stony coral *Pocillopora damicornis* has nearly 2×10^7 vibrio cells per cm^3 of infected tissue, whereas healthy tissues of the same coral have only 6×10^6 vibrio cells cm^{-3} (Luna *et al*., 2007). One hundred and thirty-one vibrio isolates were obtained from mucus of 22 coral specimens using TCBS medium (Table S1 and S2). *Vibrio alginolyticus*, *V. harveyi*, *V. harveyi*-like, *V. campbellii*, *V. coralliilyticus*, *V. tubiashii*, *V. rotiferianus* and *V. sinaloensis* were the most frequently retrieved vibrio species (Fig. 1). A vibrio species is defined as a group of isolates that share more than 97% *pyrH* sequence similarity (Thompson *et al*., 2007; 2008). *Vibrio alginolyticus* and *V. harveyi* appeared in *M. hispida*, *M. braziliensis* and *P. dilatata*. *Vibrio tubiashii* and *V. rotiferianus* appeared associated with *M. braziliensis* and *P. dilatata*, while *V. sinaloensis* appeared associated only with healthy *M. braziliensis*. According to our analysis, diseased corals did not possess a different vibrio microbiota. For instance, the species *V. coralliilyticus*, *V. harveyi*-like and *V. alginolyticus* encompassed strains originated from both apparently healthy and diseased corals. This fact may suggest that not all strains of a given species are pathogenic or have different levels of virulence. The fact that the vibrio core group (i.e. *V. alginolyticus*, *V. rotiferianus*, *V. harveyi* and *V. campbellii*) was the most prevalent in all coral species suggests an important role within the coral holobiont, especially because some of these vibrios are putative N_2 fixers (Chimetto *et al*., 2008). In a previous study of corals sampled from the Southern of Brazil, a similar pattern was found, with the predominance of the vibrio core group in *M. hispida*

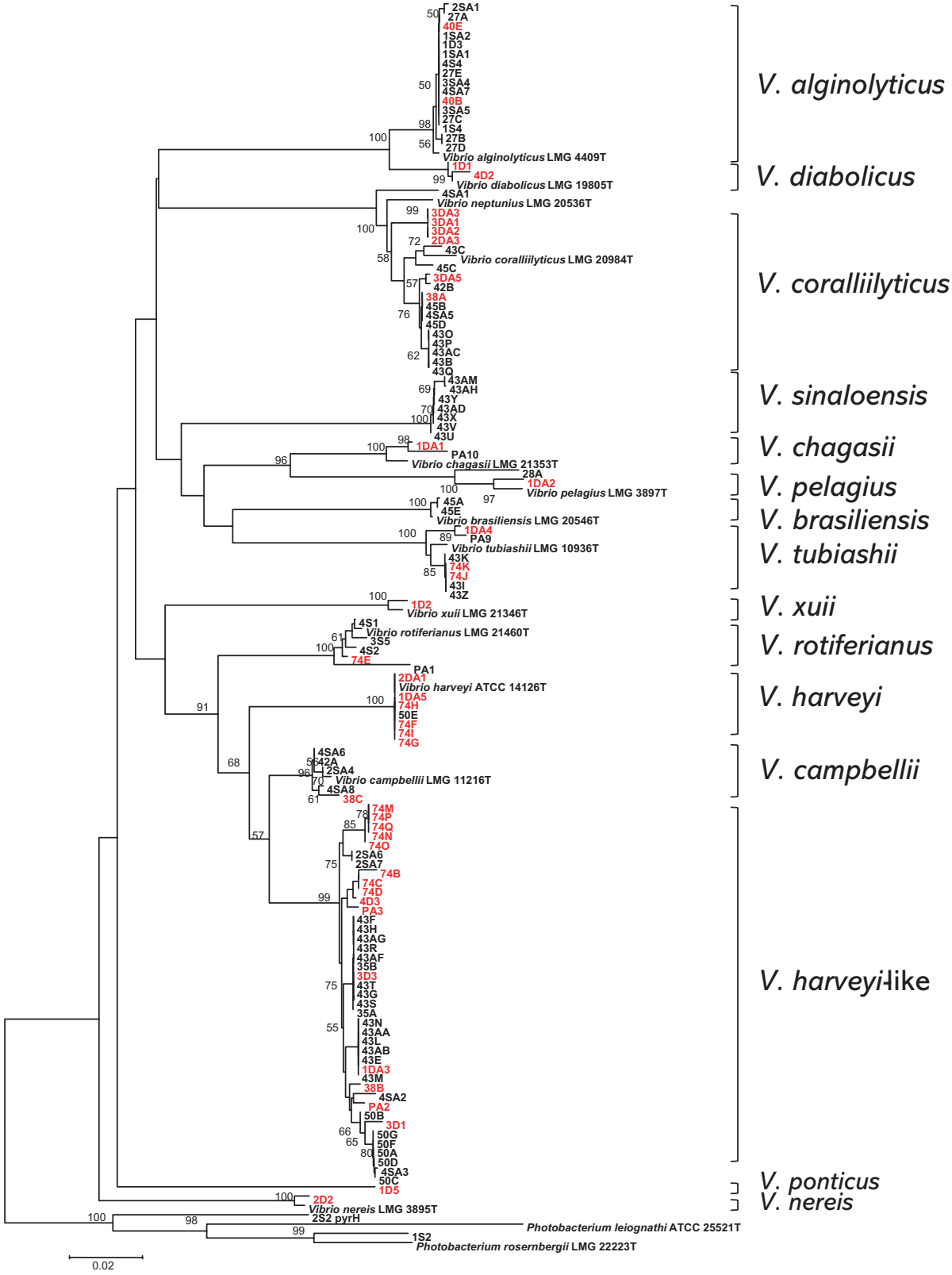

Fig. 1. Diversity of vibrios. Phylogenetic tree based on the *pyrH* sequences using the Neighbour-Joining method. *pyrH* sequences of type strains retrieved from our own database were included. Scale bar indicate 2% nt divergence. Strains in red were isolated from diseased corals, whereas strains in black were isolated from healthy corals.

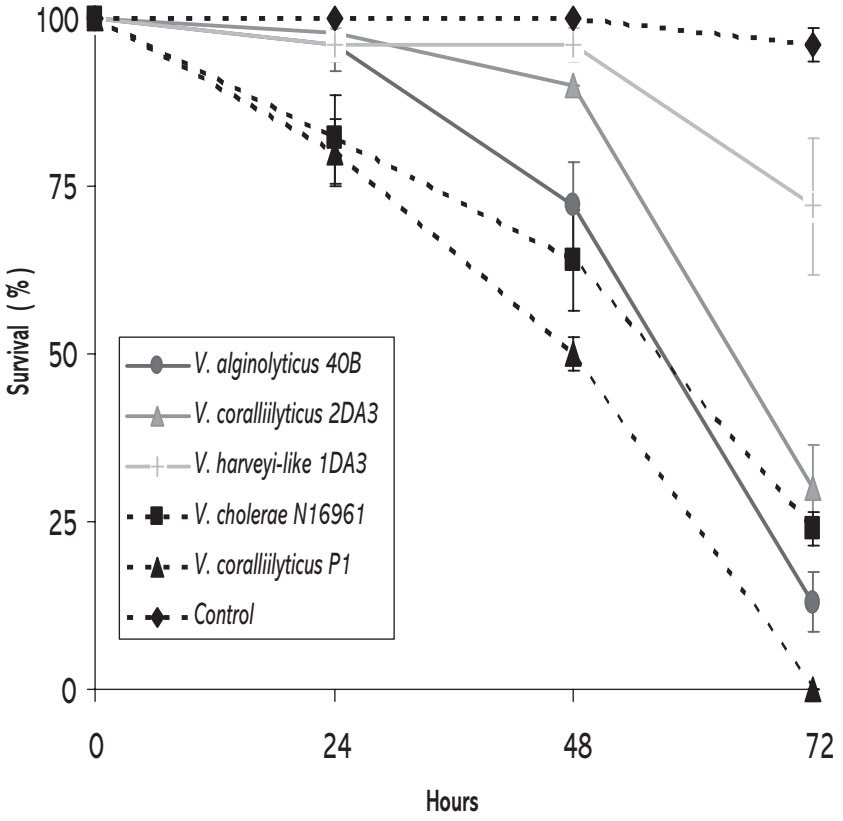

Fig. 2. Pathogenicity of selected vibrio strains. Means and standard deviations of five replicates. *Vibrio cholerae* N16961 and *V. coralliilyticus* P1 are included as a positive controls, whereas the control treatment does not contain bacteria.

and in zoanthids with the co-occurrence of a high number of populations of *V. harveyi* and *V. alginolyticus* (Chimetto *et al*., 2009). If these vibrios indeed fix nitrogen in the holobiont, the resultant availability of ammonium might have a negative effect in the holobiont. When a certain concentration is exceeded, ammonia (its non-ionized form; NH_3) might be toxic for both zooxanthellae and the coral host. If we consider that the vibrio core group represents roughly half of all the vibrio community, we might conclude that their abundance is around 10^5 cfu ml^{-1} in mucus of the coral *Mussismilia* spp. The average total abundance of mucus bacterial microbiota of *Mussismilia* spp. is 1.9×10^7 cfu ml^{-1} according to flow citometry (F.L. Thompson and R. Paranhos, unpubl. data), suggesting moderate abundance of vibrios. However, these bacteria can grow rapidly and overcome other members of the bacterial community under stressful environmental conditions (e.g. high temperature and high nutrient loads). Under such circumstances, the growth of potentially pathogenic vibrio strains of the core group might lead to coral disease.

Three representative vibrio isolates (*V. alginolyticus* 40B, *V. harveyi*-like 1DA3 and *V. coralliilyticus* 2DA3) were selected in order to evaluate their pathogenic potential in a model organism. These isolates represent the vibrio species most frequently retrieved in this study. Pathogenicity tests showed different virulence levels in *Drosophila* for each vibrio isolate (Fig. 2). Flies in the control treatment had an average survival of 96% after 3 days, whereas on average 76% and 100% of the flies died in the control treatments containing *Vibrio cholerae* N16961 and *V. coralliilyticus* P1. *Vibrio alginolyticus* 40B caused the highest virulence among the Brazilian coral isolates ($P < 0.001$) (88% average mortality after 3 days). On the other hand, *V. harveyi*-like 1DA3 isolate caused the lowest mortality (*c*. 25% average mortality; $P < 0.001$). Clearly, *V. coralliilyticus* 2DA3 caused a lower mortality than *V. coralliilyticus* P1 already after 24 h of infection, suggesting that strains from the same species can have different levels of virulence in a model animal. The results presented in the study demonstrate unambiguously that vibrios isolated from corals have the potential to kill the model animal under controlled conditions. We do not imply that findings obtained in the lab can be transferred to the field, but the challenge tests point to virulence of selected isolates of vibrios. We used *V. coralliilyticus* P1 as a control in our pathogenicity experiments because it is a proven coral pathogen (Sussman *et al*., 2009). *Vibrio coralliilyticus* P1 was shown to be highly virulent to different coral species, including *Pachyseris speciosa*, *Montipora aequituberculata* and *Acropora millepora* (Sussman *et al*., 2008). In present study, P1 killed approximately 25% of the model animal in 24 h and 100% in 72 h, suggesting that the results in the fly model may relate to

coral pathogenicity. *Vibrio harveyi*-like 1DA3 caused only a moderate fly mortality though. There is not a precise explanation for this phenomenon, but virulence factors produced by *V. harveyi*-like 1DA3 may be temperature-dependent and thus would be produced at higher temperatures. Another possibility is that this vibrio has indeed a lower virulence due to the lack of some virulence genes. The super-natant of the three coral vibrio isolates (which were produced overnight in cultures grown at 25°C) caused low fly mortality (< 10%) after 72 h, suggesting that these vibrios need to be in contact with the host cells in order to secret the toxins directly into the flies. *Vibrio cholerae* infects and kills flies by means of a cholera-like disease mediated mainly by the cholera toxin (Blow *et al*., 2005). Vibrios may kill the flies by means of the production of different types of virulence factors, e.g. proteases, lipases and haemolysins. Both *V. alginolyticus* 40B and *V. harveyi*-like 1DA3 showed high proteolytic activity on agar plates. Additional work is needed in order to determine the exact virulence factors responsible for pathogenicity in flies and in corals.

This is a first survey aiming at the characterization of the culturable bacteria from the Brazilian-endemic corals (*M. braziliensis*, *M. hispida* and *P. dilatata*) endangered of extinction. Our results revealed that these corals are reservoirs of the potentially virulent strains of vibrios. This study underpins ongoing research focused on the study of the bacterial (vibrio) ecology in the Abrolhos Bank and the genetic dissection of the virulence genes responsible for coral diseases.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Study area.
Fig. S2. Healthy (A–C) and diseased (D–F) representative specimens of *Mussismilia braziliensis*, *M. hispida* and *P. dilatata* respectively.
Fig. S3. Nutrient concentration in seawater. Chlorophyll-*a*, pheophytin and MOD are in $\mu g\ l^{-1}$, whereas inorganic orthophosphate and inorganic nitrogen (ammonia, nitrite and nitrate) are expressed in μM.
Fig. S4. Vibrios counts (cfu per ml of water and mucus formed in TCBS).
Fig. S5. Diversity of heterotrophic bacteria. Phylogenetic tree based on the 16S rRNA sequences using the Neighbour-Joining method. 16S rRNA sequences of type strains retrieved from GenBank were included. Scale bar indicate 5% nt divergence. Strains in red were isolated from diseased corals, whereas strains in black were isolated from healthy corals.
Table S1. Simplified strain list.
Table S2. Detailed strain list.
Appendix S1. Experimental procedures.

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