



## Role of bacterial communities in coral's defence against a causative agent of coral bleaching: *Vibrio coralliilyticus*

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### ABSTRACT

**Aims:** Different studies have shown that members of the *Vibrio* such as *Vibrio coralliilyticus* and *Vibrio shiloi* are opportunistic pathogens which can cause coral lysis. The aims of this study were to assess whether this results of the virulence of *V. coralliilyticus* are transmittable to *Acropora hyacinthus* and *Porites lobata*, and what role the microbiome of the corals plays during exposure to *V. coralliilyticus*.

**Methodology and results:** In laboratory-based experiments, we examined the impact of *V. coralliilyticus* (ATCC BAA-450) to the microbiome of *Acropora hyacinthus* and *Porites lobata*. *A. hyacinthus* and *P. lobata* were exposed to ampicillin, *V. coralliilyticus*, and a combination of both. Results indicate a resistance of *A. hyacinthus* to *V. coralliilyticus* through the microbiome and underpin the importance of the microbiome for the coral's health.

**Conclusion, significance and impact study:** Further studies are needed to identify the bacteria responsible for the coral resistance and could in future lead to the development of a probiotic treatment or prevention of bleaching for sensitive corals.

**Keywords:** coral bleaching, microbiome, *Vibrio coralliilyticus*, *Acropora hyacinthus*, *Porites lobata*

### INTRODUCTION

Corals harbour endosymbiotic intracellular single-celled algae called zooxanthellae, which perform photosynthesis and produce nutrients for the coral (Douglas, 2003). Under stress, zooxanthellae release toxic substances and the corals exclude the zooxanthella (Banin *et al.*, 2000) in a process called coral bleaching. In the last decades, there has been an increase of coral bleaching. Several mass bleaching events have occurred; including 1998 (Aronson *et al.*, 2000) and 2010 (Eakin *et al.*, 2010) and a third global mass bleaching is currently under way. Such mass bleaching events correlate with warm seawater periods (Hoegh-Guldberg, 1999) and it is feared that, with global warming, the mass bleaching events may occur more often and with higher severity (Kimes *et al.*, 2011).

Other causes for coral bleaching are thermal shock (heat shock and cold shock), high UV irradiance, prolonged darkness, exposure to heavy metals and/or pathogenic bacteria (Douglas, 2003). While there is a correlation between high seawater temperature and coral bleaching, it is not absolute, and environmental stress alone is insufficient (Banin *et al.*, 2000). In *Oculina patagonica* - a model organism for bleaching studies - the bacterium *V. shiloi* was reported to be the causative agent

for coral bleaching (Kushmaro *et al.*, 1996). The temperature-dependent bacterium adheres to a  $\beta$ -D-galactopyranoside-containing receptor on the coral surface (Toren *et al.*, 1998), penetrates the epidermal layer and differentiates into a viable but not culturable (VBNC) state and multiplies intracellularly, reaching densities up to  $10^9$  cells/cm<sup>3</sup> (Banin *et al.*, 2000). At a temperature between 20-32 °C, with a maximum at 28-29 °C (Banin *et al.*, 2000), *V. shiloi* produces a peptide exotoxin that inhibits algal photosynthesis and causes bleaching (Ben-Haim *et al.*, 1999). Ben-Haim (2003) found another temperature-dependent coral pathogen - *V. coralliilyticus*. The hypothesis of bacterial infection as the main cause for bleaching remains, however, controversial and may not be valid for all species of corals (Ainsworth *et al.*, 2007).

Between 2002 and 2004, *O. patagonica* became resistant to *V. shiloi* (Mills *et al.*, 2013). Due to the lack of an adaptive immune system, it was suggested that other symbiotic microorganisms (Rohwer *et al.*, 2002) caused the resistance (probiotic hypothesis). In support of the probiotic hypothesis, resistant corals became sensitive towards *V. shiloi* after an antibiotic treatment which had killed the coral-associated microbiome (Mills *et al.*, 2013). While it is still unclear whether *Vibrio* sp. is a primary or

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opportunistic pathogen, Mills *et al.* (2013) showed that antibiotics can prevent healthy corals from bleaching when the water temperature increases. The change in water temperature does not only affect potential coral pathogens but also the coral-associated microbes. Kuek *et al.* (2015) found that the ability of bacteria living in coral mucus to inhibit *Vibrio* sp. changed at different temperatures and that potentially invasive bacteria exhibited higher activity at elevated temperatures.

The water temperature surrounding the reefs in Talang-Satang National Park range between 28 - 31 °C throughout the year, however coral assemblages are healthy and diverse. Although the average seawater temperature is around the optimum of the toxin P production of *V. shiloi* and bacterial isolates are found, no bleaching is observed in the local reefs (Kuek *et al.*, 2015, 2016). A possible explanation for this phenomenon might be the existence of a probiotic microbiome. Thus, the aims of this study were to assess (i) whether the results of the virulence of *V. coralliilyticus* are transmittable to *A. hyacinthus* and *P. lobata*, and (ii) what role the microbiome of the corals plays during exposure to *V. coralliilyticus*.

## MATERIALS AND METHODS

### Obtaining and maintenance of the corals

Fragments of *A. hyacinthus* and *P. lobata* were collected using a hammer at Satang island (N 1°46' 52.7", E 110°10' 8.1"). The corals were maintained in a 240 L aquarium with an air pump, a protein skimmer and a UV disinfection filter. The temperature of the aquarium was set to 27 °C using a chiller. The water in the aquaria was made by adding Instant Ocean sea salt (SS15-10) to sterile Milli-Q water. Corals were adapted to the aquaria for at least one week. To perform the experiments, corals were broken into pieces of 1 – 4 cm<sup>3</sup> (*A. hyacinthus*) and 4 – 6 cm<sup>3</sup> (*P. lobata*) and left for three days to recover. Afterwards, fragments were distributed randomly to smaller aquaria (2 L each) without a filtration unit, but with an air pump each and a light dark circle (mercury vapour lamp) of 12 to 12 h. For the small tank, water was obtained from the big tank. Temperatures were measured daily by thermometer.

### Mucus and tissue isolation from corals

To examine changes in the microbiome, coral mucus and tissue were separated from the corals. For the extraction of mucus, corals were incubated in empty tubes on ice for 2 h to stress them and the mucus produced was collected. A high pressure spray gun (F-75G from Muzi) was used to remove the tissue off the corals.

### Pathogenic bacteria and infection experiments

*V. coralliilyticus* (ATCC BAA-450) was maintained at 25 °C in MB medium (Marine broth Zobell, M385 from HiMedia laboratories) or on MB agar (Marine agar Zobell,

M384 from HiMedia laboratories). Experiments were conducted with overnight cultures of the bacterium. Occurrence of *Vibrio* sp. in the coral samples was assessed using a selective medium, Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar. To assess the effect of an *V. coralliilyticus* infection to *A. hyacinthus* and *P. lobata*, we randomly distributed healthy fragments of the corals to 8 aquaria (4 with 7 *P. lobata* fragments each and 4 with 7 *A. hyacinthus* fragments each). One day prior to the infection (day 0), we treated half of the tanks with 50 µg/L ampicillin to kill the coral-associated microbiome. After this treatment, we took 1 coral from each aquarium for further examination. At day 1 (all corals were still 100% healthy), half of the aquaria were infected by adding *V. coralliilyticus* suspension to the aquaria (final concentration 200 × 10<sup>6</sup> bacteria/mL per aquarium). Thus, for every species there were four different treated aquaria: one without any treatment, one treated with *V. coralliilyticus*, one with ampicillin and one with both *V. coralliilyticus* and ampicillin.

### Changes in the microbiome of the corals

Changes in the culturable bacterial community associated with the corals (isolated from tissue and mucus) were monitored using spread plates (half-strength Marine Agar (MB) and *Vibrio* sp. specific TCBS agar). Colony forming units (CFU) were monitored and CFU on MB agar considered as total amount of bacteria, while CFU on TCBS considered as *Vibrio* sp.. The DNA of cultured bacteria was isolated using the Freeze and Thaw method and amplifications of bacterial 16S rRNA genes performed with broad-specificity primers 8F (Eden *et al.*, 1991) and 519R (Lane *et al.*, 1985). Thermal cycle programmed for 5 min at 95 °C as initial denaturation, followed by 30 cycles of 30 sec at 95 °C for denaturation, 30 sec at 55 °C as annealing, 30 sec at 72 °C for extension, and final extension at 72 °C for 5 min. The PCR product was sent to 1st BASE for sequencing. The sequences were identified by using BlastN on the NCBI nucleotide database.

### Statistical analyses

All statistical analyses were done with the statistical software R (Version 3.01). As a normal distribution is not assumed, a Mann-Whitney U test was used to test for statistically significant differences.

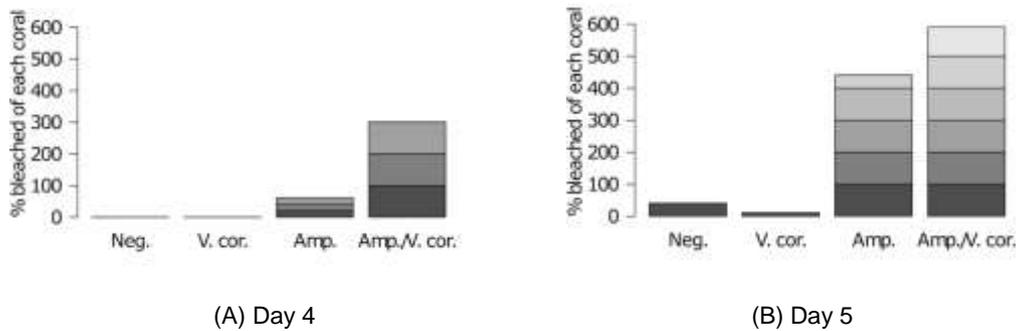
## RESULTS

### Effects on the corals after exposure to *V. coralliilyticus*

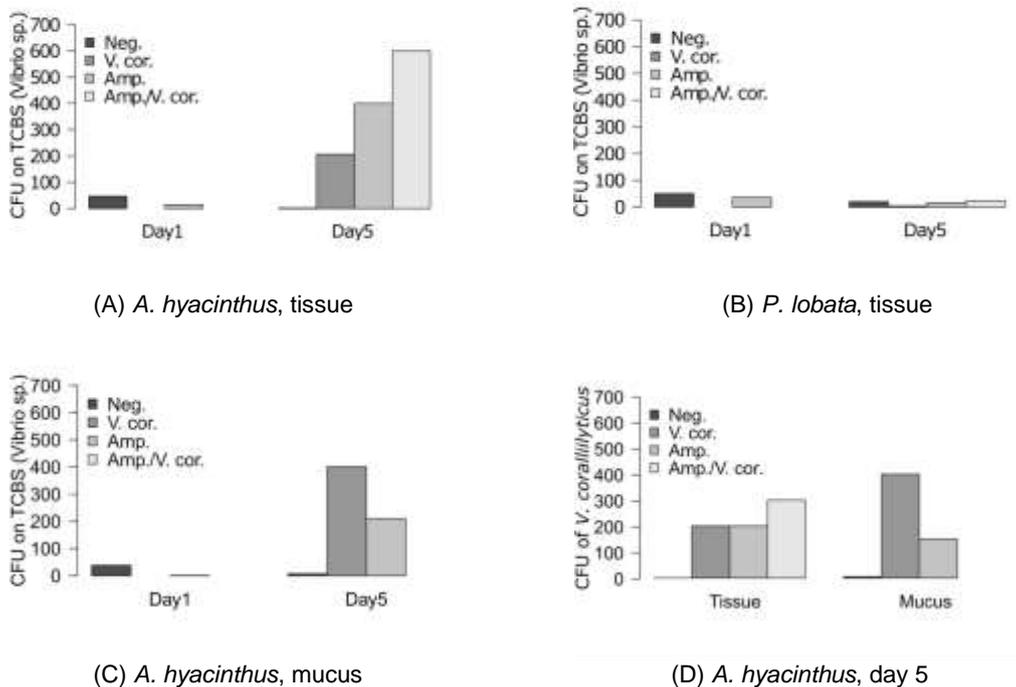
The first two days after adding the bacteria, the corals showed no evidence of bleaching. At day 4, we could observe that half of the *A. hyacinthus* were bleached in the aquarium with *V. coralliilyticus* and ampicillin. The aquarium treated only with ampicillin showed first indications of bleaching (Figure 1a). At day 5, all 6 *A.*

*hyacinthus* in the *V. coralliilyticus* and ampicillin aquaria were bleached completely (only one showed a little rest of tissue), from the ampicillin treated aquaria 4 corals were bleached completely, 1 was still bleaching, and 1 healthy (Figure 1b). All the other fragments of *A. hyacinthus* were near optimum health. *P. lobata* were completely healthy under all tested conditions. The Mann-Whitney U test

shows a significant ( $p < 0.05$ ) difference between treatment with *V. coralliilyticus* and ampicillin/*V. coralliilyticus* as well as between the negative and the ampicillin treated corals. Between the ampicillin treatment with and without *V. coralliilyticus* infection the Mann-Whitney U test shows no significant difference ( $p = 0.227$ ).



**Figure 1:** Health status of the *A. hyacinthus* A, four and B, five days after treatment start. Each sub bar represents the percentage of the bleaching of one coral, the stacked bars show the combined percentage of all six corals (maximum 600%) per treatment. The corals were treated with *V. coralliilyticus* ( $200 \times 10^6$  bacteria/mL), ampicillin (50  $\mu$ g/mL), both or nothing.



**Figure 2:** Colony forming units (CFU) on TCBS agar (specific for *Vibrio* sp.) in samples of the infection experiment. A, and B, show the CFU, isolated from tissue, on day 1 (before adding  $200 \times 10^6$  *V. coralliilyticus* per mL water, but one day treated with 50  $\mu$ g/mL ampicillin) and day 5 (four days treated with the *V. coralliilyticus* and five days with the ampicillin). C, shows the CFU on TCBS agar isolated from mucus and tissue of *A. hyacinthus* on day 5. D, shows the CFU of *V. coralliilyticus* (identified by morphology and confirmed with sequencing) on TCBS, isolated from tissue of *A. hyacinthus* on day 5.

### Changes of microbiome of coral tissue and mucus during infection

Data from the MB agar spread plates has to be interpreted with caution as many of the plates were overgrown (90 – 400 colony forming units [CFU] per plate). The results clearly indicate that the *A. hyacinthus* tissue contains a higher number of bacteria after the ampicillin treatment (250 CFU) as well as after the ampicillin and *V. coralliilyticus* treatment (400 CFU) compared to the negative control (100 CFU) and the *V. coralliilyticus* treated corals (90 CFU).

CFU counts on TCBS agar (*Vibrio* sp. counts) showed an increase in *A. hyacinthus* tissue after treatments with *V. coralliilyticus*, ampicillin or both (Figure 2a). CFU counts were higher for *Vibrio* sp. after the ampicillin treatment than the *V. coralliilyticus* treatment, and the combination of both showed the highest CFU counts on TCBS agar. No increase in CFU counts on TCBS agar

were observed for *P. lobata* (Figure 2b).

The mucus samples of *A. hyacinthus* showed higher CFU counts for *Vibrio* sp. in the *V. coralliilyticus* treated corals compared to the ampicillin treated ones. Results for the tissue samples showed the opposite results as discussed above (Figure 2c). We were not able to isolate mucus from the corals treated with both ampicillin and *V. coralliilyticus*, because all corals were bleached and unable to produce mucus. Also, *P. lobata* produced almost no mucus, so we were not able to isolate bacteria from its mucus, neither on MB, nor on TCBS agar.

We were further able to identify *V. coralliilyticus*, based on their morphology on TCBS and MB agar. Identification was confirmed by DNA sequencing (Table 1) and therefore allowed distinguishing between *V. coralliilyticus* and *Vibrio* sp.. For the treated corals, there was an increase of *V. coralliilyticus* (Figure 2d) and *V. shiloi*. There were little other *Vibrio* sp. found in the coral, but for those no increase was observed.

**Table 1:** Best hits of the sequence of the control and isolated strains (four colonies) in the NCBI nucleotide database by BLASTn algorithm. The replicas are not shown because they were redundant.

Sample	Hit	Accession	Ident	e value
Control <i>V. cor</i>	<i>Vibrio coralliilyticus</i> stra...	CP009617.1	99%	0.0
Isolate <i>V. cor</i>	<i>Vibrio coralliilyticus</i> stra...	CP009617.1	98%	0.0

### DISCUSSION

Our results show that the bacterial community of *A. hyacinthus* and *P. lobata* play an important role in their health. Both seemed to be able to resist infection by *V. coralliilyticus* when healthy. While *P. lobata* remained resistant even after its microbiome was obliterated by ampicillin, *A. hyacinthus* became sensitive to *V. coralliilyticus* after the ampicillin treatment. Several studies including Kuek *et al.* (2015) have stated that coral mucus exhibit antimicrobial properties. Therefore, it is possible the *P. lobata* harbours a group of bacteria that are resistant to ampicillin. Additionally, It has been shown that genetic aspects can lead to a resistance against coral pathogenic bacteria (Dixon *et al.*, 2015) and this may be another possible explanation for its observed resistance *V. coralliilyticus*. *P. lobata* seems to be a coral species well adapted to environmental changes and stresses.

Conversely, *A. hyacinthus* turned sensitive to *V. coralliilyticus* after ampicillin treatment. While more in-depth studies are needed to confirm the route of infection, our data support the notion that *V. coralliilyticus* was the cause for the observed bleaching. CFU counts of *V. coralliilyticus* increased rapidly in *A. hyacinthus* when the coral microbiome was weakened by addition of ampicillin and corals treated with ampicillin and *V. coralliilyticus* bleached faster than the ones treated with ampicillin only. Our data might help to explain why corals in the Satang-Talang National Park do not bleach even when the surrounding average seawater temperature is considered too high. It seems that the corals in the local reefs are resistant to *V. coralliilyticus* infection as long as their microbiomes are not disrupted. This finding also highlights

the potential of developing a probiotic treatment for corals to prevent infection by bacterial pathogens.

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