

Microbial communities associated with skeletal tumors on *Porites compressa*

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Abstract

Coral tumors are atypical skeletal forms found on coral reefs worldwide. Here we present an analysis of the microbial communities associated with skeletal tumors on the coral *Porites compressa*. Microbial growth rates on both healthy and tumorous *P. compressa* were decoupled from the surrounding water column. Microbial communities associated with tumorous colonies had a significantly faster growth rate than those associated with healthy *P. compressa*. The microbial community associated with the tumors contained more culturable *Vibrio* spp. and could utilize more carbon sources than the microbes associated with healthy colonies. Presence of tumors affected the composition and dynamics of the microbial population associated with the entire colony.

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1. Introduction

Coral tumors are abnormal growths that lead to enlarged skeletal elements [1–3]. Skeletal tumors on corals show atypical and rapid growth [3,4]. Most tumors are characterized by fewer numbers of polyps per surface area, finer skeletal structures than normal, and a marked

reduction of zooxanthellae [1,3,4]. Normal polyp structures (e.g., tentacles, mesenteries) are often reduced or absent within coral tumors [2]. With the exception of one case in which algae were associated with the tumors, most studies have been unable to identify exogenous organisms or intracellular infection by parasitic agents [2,3,5]. It has been suggested that tumor formation and growth may be influenced by environmental factors, however, no clear correlation has been established [2]. Coral tumors do not appear to be transmitted between colonies, even after fusion of healthy and tumor fragments [3].

Since they were first described over 30 years ago, tumors have been observed on many coral species from

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more than 10 families [2,4,6]. Coral tumors are widespread and have been recorded on reefs worldwide. In a recent survey at 18 sites around Oahu, Hawaii, growth anomalies on four different *Porites* spp. were found at 44% of the sites (Aeby et al., unpublished data). Tumors reduce the overall reproductive potential of the colony, make the coral more susceptible to bleaching, and create a thin epidermis that can be more easily invaded by other organisms [1,2].

Several studies have suggested that even the apparently healthy part of a coral colony with tumors is affected by the tumor. For example, rapid growth of tumors is accommodated by the reduction of growth in healthy parts of the same colony [4,7] and Yamashiro et al. showed depletion of storage lipid in the tumorous coral tissue, as is seen in mammalian tumors. The lipid depletion may be explained by an increased energy demand in the coral tissue for tumor synthesis [7]. This suggests nutrient and energy translocation from the nearby normal tissue to the tumors through the gastro-vascular system that connects the polyps [4].

Recent studies have demonstrated that Bacteria and Archaea are diverse and abundant on reef-building corals [8–11]. Using fluorescence in situ hybridization (FISH) with domain-specific peptide nucleic acid (PNA) probes, Wegley et al. showed that there are $\sim 10^8$ microbial cells per cm^2 of *Porites* spp. [8]. Analyses of carbon source utilization patterns by microbes cultured from coral mucus layers have suggested that corals have unique and species-specific microbial communities [12,13]. Sequence-based approaches have demonstrated that specific coral–bacteria associations are maintained over time and space [9,10]. In addition, Rohwer et al. showed that bacterial ribotypes can be spatially structured within colonies [10]. The specific and structured association of microbes with corals suggests that microbes play an important role in coral health and ecology.

Here we present an analysis of the microbial communities associated with skeletal tumors in the reef-building coral *Porites compressa*. The microbial community associated with the tumors differed in composition and grew more rapidly than the microbes associated with healthy *P. compressa*. Presence of a tumor altered the microbial activity on the entire colony. This microbiological approach of studying coral tumors provides insight into the effects of coral tumors on the entire coral holobiont.

2. Materials and methods

2.1. Collection of coral and water samples

Coral samples were collected from the lagoon and reef crest of Coconut Island (Oahu, Hawaii; Fig. 1(a)) using a hammer and chisel. At the reef crest, samples

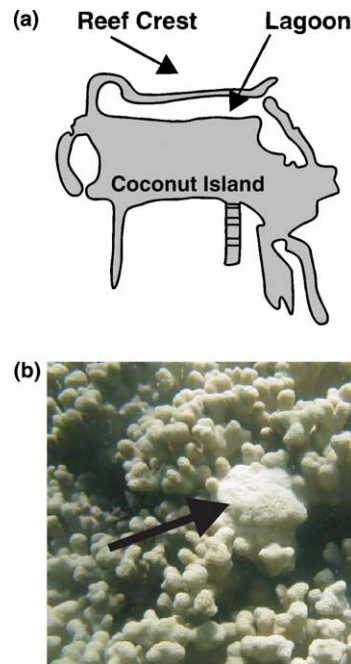


Fig. 1. (a) Schematic of Coconut Island; Oahu, Hawaii. Coral samples were taken either from the lagoon or the reef crest. (b) Example of a tumor on *Porites compressa*. The arrow indicates the tumor.

were taken from tumors on *P. compressa* ($n = 10$), apparently healthy parts of tumorous colonies ($n = 10$), and remote healthy colonies ($n = 6$). The samples were collected from random locations and tumorous colonies were spatially interspersed with healthy colonies. An example of a tumor on *P. compressa* is shown in Fig. 1(b). Remote healthy colonies were also harvested from the lagoon ($n = 10$). The coral samples were immediately placed in individual Ziploc bags and brought to the surface for further processing. Water samples were collected in 50 ml conical tubes halfway between the randomly selected coral colonies and the surface.

2.2. Measuring microbial production

Within 15 min of bringing the corals to the surface, corals were placed in 0.2 μm filtered bacteria-free seawater and the mucus layer was removed using a syringe. The mucus was transferred to a 15 ml conical tube, shaken vigorously, and then 1.5 ml aliquots were immediately placed in microfuge tubes containing 25 nM of [methyl- ^3H]-thymidine (Amersham Biosciences; Buckinghamshire, UK). The leftover mucus/sterile-seawater mixture was used for the direct counts and culturing (described below). An aliquot was also added to ‘‘Carry-over Control’’ tubes that contained 75 μl 100% trichloroacetic acid (TCA) in addition to the [methyl- ^3H]-thymidine. The Carry-over Controls were used to determine the amount of unincorporated [methyl- ^3H]-

thymidine that was still found in the samples after processing in the absence of cellular assimilation. Samples and Carry-over Controls were incubated at room temperature for 1 h. The reaction was stopped by adding 75 μ l of 100% TCA and incubated on ice for 10 min. The TCA precipitates were pelleted in a microfuge (10 min; 4 °C; 15,000g). The supernatant was aspirated away and the pellet washed with \sim 1.5 ml of ice cold 5% TCA. This was pelleted again in the microfuge and then washed with \sim 1.5 ml of ice cold 80% ethanol. The pellet was resuspended into 500 μ l bleach and vortexed until completely resuspended. This mixture was transferred to 20 ml scintillation fluid and counted. All samples were done in triplicate.

2.3. Direct counts of prokaryotes in coral mucus

Ten microlitres of the mucus/sterile-seawater was diluted into 3 ml sterile seawater. This mixture was filtered onto a 0.02 μ m Anodisc, stained with 1X SYBR Gold (Molecular Probes; Eugene, OR), and microbial cells were counted using epifluorescent microscopy.

2.4. Production calculations and statistical analyses

The counting efficiency of the scintillation counter was determined to be 85%. This was used to calculate pmol thymidine incorporated into DNA $\text{ml}^{-1} \text{h}^{-1}$ ([adjustment for counting efficiency; 1 dpm per 0.85 cpm] \times [1 Ci per 2.22×10^{12} dpm] \times [1 mmol per 84 Ci]). Each sample was counted for 3 min. For each sample the three replicate counts were averaged and “Carry-over Controls” were subtracted from this value to account for unincorporated radiation. This value was converted to pmol thymidine incorporated $\text{cell}^{-1} \text{h}^{-1}$, using the mean direct count data.

The production values were then compared to determine if there were statistical differences between the growth rates of the microbes from the various corals. All treatments were compared to each other in a pair-

wise fashion using a one-tailed Mann–Whitney *U*-test for unmatched samples (Table 1).

2.5. EcoPlates

One hundred microlitres of the mucus/sterile-seawater mixture was pipetted into each well of an EcoPlate (Biolog; Hayward, CA). Three remote healthy colonies and five tumor colonies were tested to determine which carbon sources the microbial communities could utilize. Thirty-one carbon sources were tested in triplicate. Plates were scored four days after inoculation.

2.6. Culturing *Vibrio* spp.

Vibrio spp. were isolated on thiosulfate citrate bile salt sucrose (TCBS) agar (Sigma; St. Louis, MO) by spreading 100 μ l of the mucus/sterile-seawater mixture on each plate. Colonies were counted \sim 48 h later.

3. Results and discussion

3.1. Microbial growth rates on healthy *P. compressa* and in the overlying water column

Cell-specific microbial production rates on corals and in the water column were measured using [methyl- ^3H]-thymidine incorporation and direct counts. The average specific growth rate of microbes associated with healthy *P. compressa* colonies was 1.75×10^{-10} pmol thymidine $\text{cell}^{-1} \text{h}^{-1}$ (Fig. 2). The growth rates for the microbes on the healthy corals were similar to those in the overlying water column (average = 2.6×10^{-10} pmol thymidine $\text{cell}^{-1} \text{h}^{-1}$; Fig. 2). This contradicts previous studies that showed a marked increase in microbial activity on the coral surface compared to the surrounding water [14,15]. The discrepancy between these results may be due to differences in the sampling technique (see discussion below).

Table 1

Pairwise statistical comparisons of cell-specific microbial growth rates on healthy corals, corals with tumors, and in the surrounding seawater

		Lagoon		Reef crest	
		Remote colonies ($n = 10$)	Remote colonies ($n = 6$)	Tumor colonies – tumor ($n = 10$)	Tumor colonies – healthy ($n = 10$)
Lagoon	Remote colonies ($n = 10$)	na	NSD	<i>U</i> -test $p < 0.01$	<i>U</i> -test $p < 0.01$
Reef crest	Remote colonies ($n = 6$)		na	<i>U</i> -test $p < 0.025$	<i>U</i> -test $p < 0.01$
	Tumor colonies – tumor ($n = 10$)			na	NSD
	Tumor colonies – healthy ($n = 10$)				na

The medians of the samples were compared using a one-tailed Mann–Whitney *U*-test for unmatched samples.

NSD = not statistically different ($p > 0.2$).

U-test = *p*-value obtained with the one-tailed Mann–Whitney *U*-test for unmatched samples to test for a difference in medians.

na = not applicable.

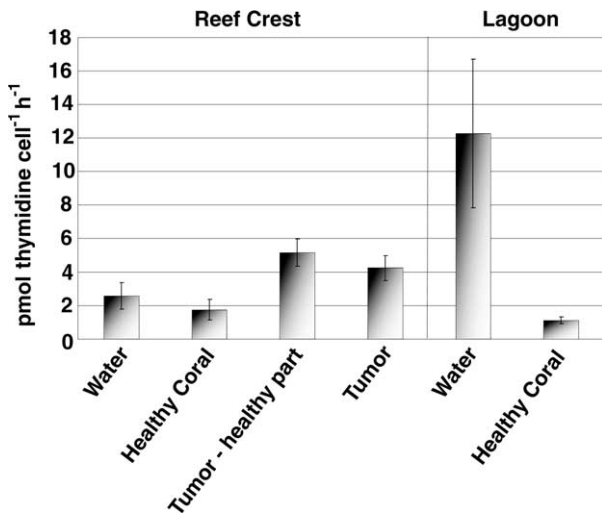


Fig. 2. Cell-specific microbial growth rates from seawater, healthy *P. compressa* from the reef crest, tumors on *P. compressa* from the reef crest, healthy areas on tumorous colonies from the reef crest, lagoon water, and healthy *P. compressa* in the lagoon. Data is shown as the mean \pm standard error for each sample.

The microbes in the lagoon water were growing much faster than those in the seawater from the reef crest (Fig. 2). The cause of this difference is not known, but it may be due to higher concentrations of dissolved organic carbon in the lagoon. All values obtained for growth rates of microbes in the lagoon water were higher than any of those observed on the corals in the lagoon. Despite the rapid growth rates of the microbes in the surrounding lagoon water, the microbial growth rates on healthy corals in the lagoon were maintained at approximately the same rate seen on healthy corals from the reef crest. This implies a decoupling of growth rates of the microbes in the water column and the microbes on the coral. Since microbial overgrowth may be dangerous for coral health, the coral's control over microbial growth rates is probably critical for maintaining the coral holobiont's health [16,17].

3.2. Microbial growth rates on healthy and tumorous *P. compressa*

The growth rates of microbes on *P. compressa* with tumors were measured and compared with the growth rates of microbes on healthy *P. compressa* (Fig. 2). The direct counts of microbes revealed that there was no significant difference in the number of microbes per surface area of the tumorous and healthy corals (data not shown). As shown in Table 1, the microbes associated with coral tumors grew significantly faster (*U*-test, $p < 0.025$) than those on remote healthy corals on the reef crest.

The growth rates of microbes found on the coral tumors were compared with those of microbes on the

apparently healthy sections of tumorous *P. compressa* colonies. The microbes associated with healthy parts of tumorous colonies grew significantly faster (*U*-test, $p < 0.01$) than those associated with remote healthy colonies (Fig. 2, Table 1). There was no significant difference (*U*-test, $p > 0.2$) in the microbial growth rates on the healthy part of tumorous colonies versus on the tumor itself (Table 1). Microbial growth rates on both the healthy part of colonies affected with tumors and on the actual tumor itself were faster than those on healthy colonies (Fig. 2). This increased microbial growth on the healthy parts of tumorous colonies indicates a whole-coral effect of the tumors, even on seemingly healthy tissues. This is consistent with data suggesting that the formation of tumors imposes an energy burden which affects the entire coral colony [4,7]. The whole-coral response has also been noted for microbial communities on diseased corals. Pantos et al. [18] showed that the bacterial community structure associated with the healthy-looking intact tissues on a white-plaque-like diseased colony of *Montastraea annularis* was substantially different from the normal microbial community associated with remote healthy colonies.

3.3. Carbon utilization profiles of microbes associated with healthy and tumorous *P. compressa*

EcoPlates (Biolog; Hayward, CA) provide a qualitative way to functionally analyze the microbial community composition in an environmental sample. Tumor colony microbes had the ability to use four more carbon sources (β -methyl-D-glucoside, L-asparagine, α -cyclodextrin, and L-threonine) than those found on healthy colonies. The microbes from the tumorous colonies could utilize an average of 16 carbon sources, while the microbes from healthy colonies could use only 10 carbon sources. This suggests a shift in the microbial community associated with the coral tumors, and that the tumor microbial community could utilize a broader range of carbon sources.

3.4. *Vibrio* spp. associated with healthy and tumorous *P. compressa*

The occurrence of *Vibrio* spp. was measured by selective culturing on thiosulfate citrate bile salt sucrose (TCBS) agar plates. Culturable *Vibrio* spp. were preferentially associated with the coral tumors. The tumor colonies contained an average of 10.2 *Vibrio* spp. colonies per 100 μ l, while the remote healthy colonies contained an average of only 0.8 *Vibrio* spp. colonies in the same volume of mucus. In addition, the healthy part of the colonies with tumors had an average of 7.6 *Vibrio* spp. per 100 μ l mucus, showing that the microbial community of the entire colony was affected by the tumor. There was no difference in the concentration of *Vibrio*

spp. found on the remote healthy corals from the reef crest and those from the lagoon (0.8 vs. 0.6 *Vibrio* spp. per 100 μ l respectively), even though there were many more *Vibrio* spp. in the lagoon water than in the seawater (12 vs. 0 *Vibrio* spp. per 100 μ l respectively). This supports our previous observations that the bacterial populations on corals are different than those in the water column, and are tightly controlled by the coral.

Although the significance of the *Vibrio* spp. on the coral colonies with tumors still remains unknown, this is an important avenue to pursue. *Vibrio* spp. have been identified as pathogens of corals, and may be an indicator of overall coral health [19–21]. This study shows that either there are more *Vibrio* spp. on corals with tumors, or that *Vibrio* spp. found on healthy *P. compressa* have shifted from an unculturable state to a culturable state on the tumorous colonies.

3.5. Methodological concerns when measuring microbial production on corals

The methodology described here for measuring microbial production on corals is a modification upon the standard sample collection. Coral mucus is typically removed from the coral underwater using a syringe, which inevitably draws in some surrounding seawater. If mucus removal is performed in situ and the microbes in the surrounding seawater are growing quickly (e.g., the lagoon water from this study), then the signal from the water microbes will overwhelm the signal from the coral-associated microbes. This is especially problematic for studies performed in tanks, where microbes in the water are growing significantly faster due to containment in the tank (Kline, Breitbart, Rohwer, unpublished data). To avoid this bias in the present study, pieces of coral were removed and brought to the surface, where they were placed into sterile seawater. Coral mucus was then removed using a syringe and processed immediately. The amount of time allowed between collecting the mucus with a syringe and starting the production incubation can also significantly alter the results. Earlier studies have allowed up to 2 h before beginning the microbial production assay [14,15], however, this time lag can lead to dramatic differences in observed production rates (Kline et al., unpublished data). It is therefore critical that mucus be removed in sterile seawater and processed immediately for microbial production studies.

There are a number of additional concerns to be considered when estimating microbial production rates based on the incorporation of tritiated thymidine. First, it is critical to perform a saturation curve to determine what concentration of labeled thymidine to use. The tritiated thymidine concentrations must be high enough so that all newly synthesized prokaryotic DNA incorporates the label, but not so high that it stimulates prokaryotic growth or can be utilized by eukaryotes. We

have performed saturation experiments on coral mucus using concentrations of tritiated thymidine ranging from 0.1 to 500 nM (Kline et al., unpublished data). The amount of incorporated label increased at concentrations up to 25 nM, and then did not change from 25 to 200 nM. At concentrations higher than 200 nM, a drastic increase in the amount of label was observed, which was likely due to incorporation by eukaryotic organisms or because the extra thymidine was feeding the microbes. Although concentrations of exogenous thymidine were not measured in this study, the saturation curve data suggest that extracellular concentrations of natural thymidine were low enough that a 25 nM addition of labeled thymidine was not subject to significant extracellular isotope dilution. In these experiments, it is also critical that the length of incubation be kept short (1 h) to ensure that the thymidine incorporation is only due to DNA synthesis by prokaryotic cells (Kline et al., unpublished data; [22]).

Fuhrman and Azam used autoradiography to show that virtually all nonphotosynthetic bacteria in seawater that are capable of taking up amino acids or glucose can also take up exogenous thymidine [22]. These data showed that most active microbes in seawater take up nanomolar levels of thymidine through a scavenging pathway. However, a similar analysis has not been performed for coral-associated microbes. The data presented here show that more radiolabeled thymidine was incorporated by the microbes associated with coral tumors than by microbes associated with healthy corals. At this point, there is still a possibility that differences in community composition could lead to differences in the rate of labeled thymidine uptake. It is currently unknown whether certain marine bacteria preferentially use the synthesis versus scavenging pathways. In addition, microbes from coral mucus might respond differently than microbes from the water column to the addition of labeled thymidine. Nonetheless, the data presented here show differences in thymidine incorporation rates of microbes in the water column, microbes associated with healthy corals, and microbes associated with coral tumors. We believe that these differences are most likely due to differences in microbial growth rates.

4. Conclusions

The microbial communities associated with tumors on *P. compressa* are significantly different from those associated with healthy *P. compressa*. Compositional changes in the coral-associated microbiota are accompanied by an increase in overall community growth rates on corals with tumors. Since microbial growth rates on healthy corals appear to be decoupled from the surrounding water column, this implies a disruption of the coral's regulation of microbial growth rate on the

tumorous colonies. Future research needs to determine if the microbial community changes as a result of tumor formation, or if a stress-induced condition alters the microbial community even before the tumor is evident.

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