Response of the Coral Associated Nitrogen Fixing Bacteria Toward Elevated Water Temperature

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Abstract: Coral reefs are among the most biologically diverse and economically important ecosystem on the planet. Despite the importance, reef habitat is being under threat from human exploitation, and its most serious stressor is increasing seawater temperature, an aftermath of global warming phenomenon. The increasing seawater temperature causes bleaching, diseases and insufficiency of nutrients of corals. Despite being surrounded by ocean waters were nutrient are in short supply, the reef ecosystem is a significant source of new nitrogen. Biological nitrogen fixation is a significant internal source of marine organism. The growth of all organism lies on the availability of mineral nutrients particularly of nitrogen (N_2). Approximately 80% of atmosphere is made of nitrogen, however, N_2 can only be available for use by organism unless it undergoes a process of nitrogen fixation. In this aspect, related literature on biological nitrogen fixation seems sparse especially on the effects of increasing seawater temperature, a well-known contributing factor of coral bleaching. In this study, an investigation was conducted on nitrogen fixing bacterial communities associated in the coral *Acropora digitifera*, exploring its responses towards elevated water temperature. The study shows that exposure to high temperature causes a drastic change in the community of nitrogen fixing bacteria which are abundant in coral mucus. These changes, is correlated with the shift in the metabolic function in coral holobiont, thus, affecting both health and resiliency of corals. Overall, the finding highlights the impact of elevated seawater temperature on the nitrogen fixing bacterial composition and its diversity as well as its effects of this on host metabolism.

Keywords: Nitrogen Fixing Bacteria, Coral Mucus, Elevated Water Temperature, Coral Health

1. Introduction

Nitrogen (N) is the building block in all life forms [6, 80]. It is vital element for organism life and survival. Prior to organisms usage of nitrogen, it undergoes biological N_2 fixation carried out by prokaryotes which includes the diverse group of bacteria commonly called *diazotrophs* [40, 80, 86, 87]. The ecological niches of diazotrophs is largely limited to the open ocean oligotrophic gyres were N availability is low and of high light intensities and O_2 concentration [35, 80]. Nitrogen is regarded as the most limiting element for biological productivity in the open sea [6, 25, 35, 80]. Recent studies on the phylogenetic diversity and distribution of *nifH* gene, the functional gene which encodes the dinitrogenase iron protein, reveals that in marine environments, nitrogen fixing microorganism vents to highly productive shelf areas including the coral holobiont [16, 53, 57, 86].

Recognized as a major contributor of newly produced Nitrogen into the oceans, shallow coral reefs habitat are aids a huge fraction of total benthic N_2 fixation to a global scale [55, 62, 80]. These array of micro-organism was measured from various reef surface including carbonate sediments, [7, 82], algal dominated reef crest, [29, 48, 49], mucus and tissue of living corals [44], coral rubbles and coral skeleton [48, 49, 82]. Notably, the condition of these fix nitrogen varies considerably, as most coral use nitrogen mainly for growth and maintenance [68, 69, 70]. However, coral reefs is highly vulnerable to multiple disturbances such as global warming which results to increase of sea water temperature [23, 27, 34, 64, 80].

Increasing seawater temperature causes thermal stress

pressure which leads alteration of the physiology of reef organism [30, 31, 39], as well as of coral associated microorganism [58].

Corals secret mucus [12, 13]. The chemical nature and quantity of mucus changes when corals are stressed [68, 69]. As a result, changes in the mucus environment damage the structure and membership of bacterial community [14, 71, 70]. Microorganisms are the fastest to respond towards disturbances [24, 25, 38]. Given that their responses are often nonlinear, they provide near real-time trajectories for coral reefs [3, 80].

Geared with the desire to attain a comprehensive knowledge and understanding on the underlying mechanism and interactions within the holobiont framework and the responses of corals to environmental change such as ocean warming, this study focused on how elevated water temperature affects the association of diazotrophic community with the scleractinian coral, *Acropora digitifera*. Specifically, the study concentrates on mucus-associated nitrogen fixing bacteria and its responses upon exposure to elevated water temperature. Also, to determine the functional role of the bacterial community, comparison on coral surface mucus between healthy and thermally stressed coral was made.

2. Methods

2.1. Coral Materials

Colonies of *A. digitifera* corals were collected at Caniogan Island (16° 17' 28.6" N, 120° 00' 44.2" E) Anda Reef complex, Pangasinan, Philippines. In-situ coral mucus was also collected to profile naturally occurring bacterial community. The coral was then transported with care in the Bolinao Marine Laboratory hatchery facility. Coral colonies was cut into nubbins using a wire cutter at about 2 to 3 inches long and attached to reef plugs using epoxy. Coral nubbins were acclimated for almost a month to insure recovery of wounds during cutting and to adapt into the hatchery environment in preparation for experimental heat stress. The hatchery tank was maintained in an ambient average temperature of 28°C, pH of 8.14 (Mettler Tolido pH meter) and salinity of 33.13 ppt (Atago-refractometer) in a flow through filtered sea water.

2.2. Heat Stress

Acclimated *A. digitifera* coral nubbins underwent heat stress by exposing to $32 \pm 1°C$ temperature as treatment and $27 \pm 1°C$ temperature as control for 10 days. Control tanks were maintain representing the cold month of the year (December to February) based on regular monitoring by the Bolinao Marine Laboratory. The set-up were conducted in a 12h light/dark cycle with irradiance of $14 \mu mol \ m^{-2} \ s^{-1}$. A low irradiance was used to reduce the potential contribution of high light intensity to the coral stress response. The coral nubbins were randomly distributed into each water conditions to avoid any possible bias of intercolony variability. The experimental system comprised of a 40L tanks plumed into flowing sand-filtered seawater with constant aeration. All tanks were operated into a closed system air-conditioned room with humidity of 25°C. Seawater temperature was manipulated with submersible thermostat heaters with additional submersible pump for equal distribution of heat into the tank. The water temperature in the tank was monitored using temperature probes (Labquip Temp (Labquest 2); Vernier software and Tech, Model-LQ2-LE) and recorded throughout the experiment using submersible water temperature data loggers (HOBOware pro. Onset, Pocasset, MA, USA). Throughout the course of the experiment, the state of the photosynthetic apparatus of the coral fragments was monitored, using a diving pulse-amplitude-modulated (PAM) fluorometer (Walz). PAM readings were taken from all coral fragments in the experimental and control tanks. Coral nubbins were collected in triplicate.

2.3. DNA Extraction

Individual coral fragments were rinsed in membrane filtered seawater (FSW) and sealed in a 50ml tube for 3 minutes to collect mucus secretions [41, 58]. DNA was extracted from 400 µl of mucus using a modified cetyltrimethylammonium bromide (CTAB) method. Briefly, samples were mixed with CTAB extraction buffer [100mM TrisCl, pH 8.0, 20mM EDTA, 2% CTAB, 1.4M NaCl, 2.5mg/ml lysozyme] and incubated at 37°C for 40 mins. After addition of 0.2% β-mercaptoethanol and 0.1mg/ml proteinase K, samples were then incubated at 60°C for 1 hr followed by chloroform fractionation and isopropanol precipitation [83]. The DNA pellet was washed with 70% ethanol and dried at room temperature. The DNA was dissolved in 1x TE buffer and stored at -20°C.

2.4. PCR Amplification

A 359bp fragment of the *nifH* gene was amplified using the nested PCR approach [87]. Briefly, to amplify the *nifH* fragment, 1µl template DNA was added to a first PCR mixture containing 1X PCR buffer, 4.0mM $MgCl_2$, 0.2mM dNTP, $40ng\mu l^{-1}$ bovine serum albumin, 2.5 units Taq polymerase, and 10 µM each of *nifH3* and *nifH4* primers. PCR cycles consisted of an initial heating step of 3 min at 94°C, followed by 28 cycles of 94°C for 45s, 57°C for 45s, and 72°C for 1 min, followed by a final extension for 10 min at 72°C. 1ul of the PCR product was subjected to a second round of PCR, identical to the first but with primers *nifH1* and *nifH2*. The nifH amplification products were separated by electrophoresis through 2.0% agarose gels and visualized with Sybr gold stain.

2.5. Denaturing Gradient Gel Electrophoresis (DGGE)

PCR amplification for DGGE was performed in a BioRad T100 thermal cycler using the bacterial 16S rRNA primers 357F-GC and 518R [60]. The PCR cycles consist of an initial heating step for 3 min at 94°C, 10 cycles of 94°C for 1 min,

65°C for 1 min, 72°C for 2 min with annealing temperature decreased by 1°C per cycle, followed by 28 cycles consisting of 94°C for 1 min, 55°C 1 min, and 72°C for 2 min, and a final extension for 10 min at 72°C. The PCR products were loaded into a 30-60% linear gradient of urea and formamide with 8% acrylamide. Gels were run in 1X TAE at 60V for 16 hours at 60°C in a DGGE apparatus (C.B.S. Scientific, DGGE-2001). After electrophoresis, the gel was stained for 30 minutes with SYBR Gold nucleic acid stain in 1x TAE buffer, rinsed and photographed with a Nikon Camera (Nikon Digital Camera D5100). Distinct bands were excised from the gel and placed in 30μl nuclease free water overnight to diffuse the DNA. The eluted DNA was re-amplified for direct sequencing using the same primers as for DGGE but without the GC clamp. PCR cycles for re-amplification include an initial heating step for 3 min at 94°C, followed by 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min followed by a final extension for 5 min at 72°C. 16S rRNA V3 PCR products were sequenced using dye terminator sequencing (BigDye Terminator v3.1 cycle) at FirstBase Laboratory, Malaysia. ImageJ was used to analyze DGGE banding patterns. Heirarchical clustering analysis of DGGE profiles was implemented in R using pvclust (Version 2.0-0, 2015) with unbiased p-values.

2.6. nifH Gene Cloning and Sequencing

nifH PCR products were cloned using the TOPO-TA cloning kit (Invitrogen) guided by the manufacturer instructions. Shortly, Amplified *nifH* from mucus samples were ligated into the TOPO-TA cloning vector. Inserts were transformed into the TOP 10 competent *Escherichia coli* cells. Transformation were plated on a Luria-Bertani (LB) media containing 50μg/ml ampicilin, 50μg/ml kanamycin and 40 mg/ml X-gal. Positive clones were selected by blue and white screening. Fifty white colonies of each mucus samples (Control (27°C), Treated (32°C), In-situ) were picked. The *nifH* insert was amplified using M13 forward and M13 reverse primers. PCR products from *nifH* clones were purified and sequenced using dye terminator

sequencing (Big Dye Terminator v3.1 cycle) at First Base Laboratory, Malaysia.

2.7. Data Analysis

Obtained *nifH* sequences were trimmed and clean-up before sequence alignment using CLC sequence viewer 6.8.1 (CLC bio A/S; www.clcbio.com) and was refined manually by visual inspection before structural analysis. Sequences were translated into operational protein units (OPUs) using the Expasy translate tool (http://web.expasy.org/translate). Phylogenetic affiliation of the *nifH* genes were determined by alignment to the nitrogenase nifH gene sequence database (http://www.css.cornell.edu/faculty/buckley/nifh.htm) and to NCBI non-redundant database (www.ncbi.nlm.nih.gov) using the BLASTn algorithm. Diversity parameters such as Shannon [75] and Simpson [78], as well as richness estimators (ACE and CHAO 1) of the *nifH* gene for each mucus samples were generated in MOTHUR [72, 73, 74].

3. Results

3.1. Coral Physiology

The coral fragments of *A. digitifera* survived collection, recovery, acclimatization and experimentation without exhibiting any visual signs of stress in the form of bleaching after 10 days of exposure to constant level of elevated temperature. In addition, comparable Fv/Fm values were observed between readings from freshly collected colonies and coral fragments during the acclimatization period (Figure 1). Fluorescence measurements are used to assess the physiological state of the coral photosynthetic symbiont. These values measure the kinetics of fluorescence rise and decay in the light-harvesting antenna of thylakoid membrane, thus querying various aspects of the state of the photosystem under different environmental conditions. A slight but significant decline in Fv/Fm was observed after 10 days of exposure to elevated temperature relative to the controls.
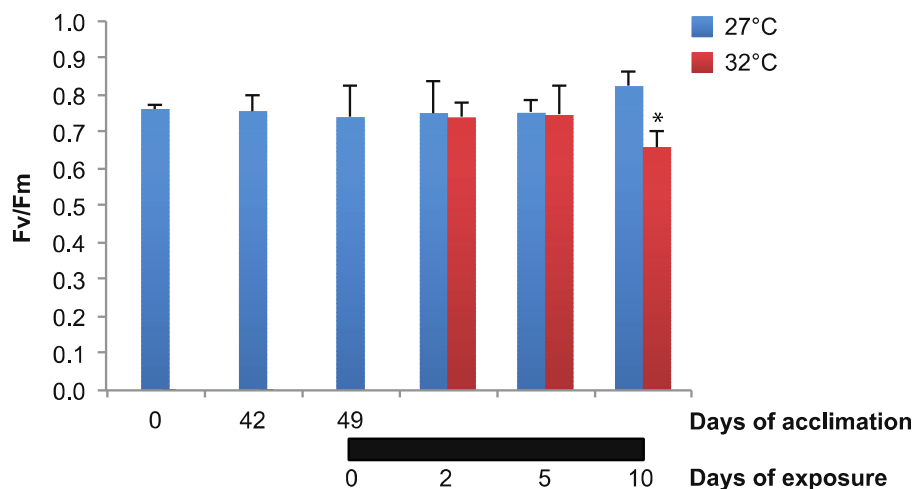

Figure 1. *Maximum dark-adapted Fv/Fm values for A. digitifera during acclimation and upon exposure to different temperature regimes. Asterisk indicates a Student's t-test p-value < 0.001.*

3.2. Shifts in Microbial Community in Response to Thermal Stress

Denaturing gradient gel electrophoresis (DGGE) was initially used to examine the bacterial community composition of the corals exposed to $27 \pm 1°C$ temperature and $32 \pm 1°C$ temperature as treatments. DGGE pattern comparison (Figure 3) showed that bacterial communities are different between coral mucus and tissue fractions and that the coral microbiota is vastly different from that of the seawater within the experimental tanks. Prolonged exposure to increased temperature resulted in the most obvious changes in the DGGE pattern for the mucus samples while tissue and seawater communities appeared to be the same. DGGE profile analysis was able to distinguish between the different coral fractions, as well as between treated and control samples (Figure 4). On the other hand, the abundance of bacteria associated with both the mucus and tissue of corals shows no change upon prolonged exposure to elevated water temperature as measured by DAPI-stained cell counting (Figure 2). However, it should be noted that these counts are only rough estimates of the number of bacterial cells. More accurate counts will be obtained with the use of bacteria specific probes.
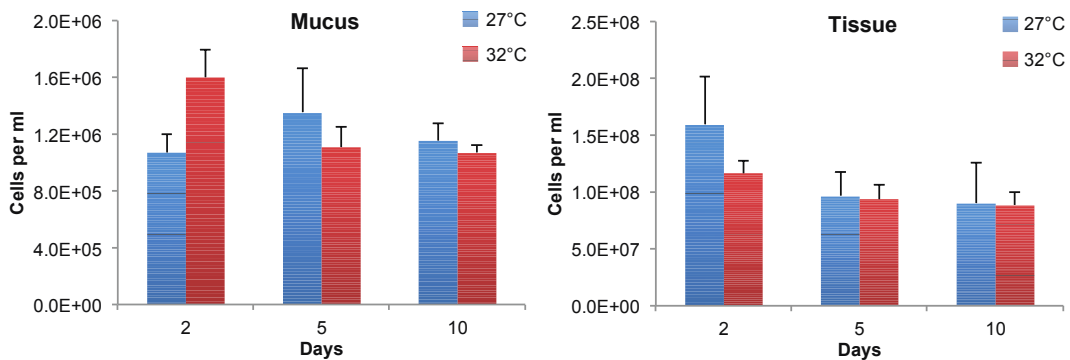

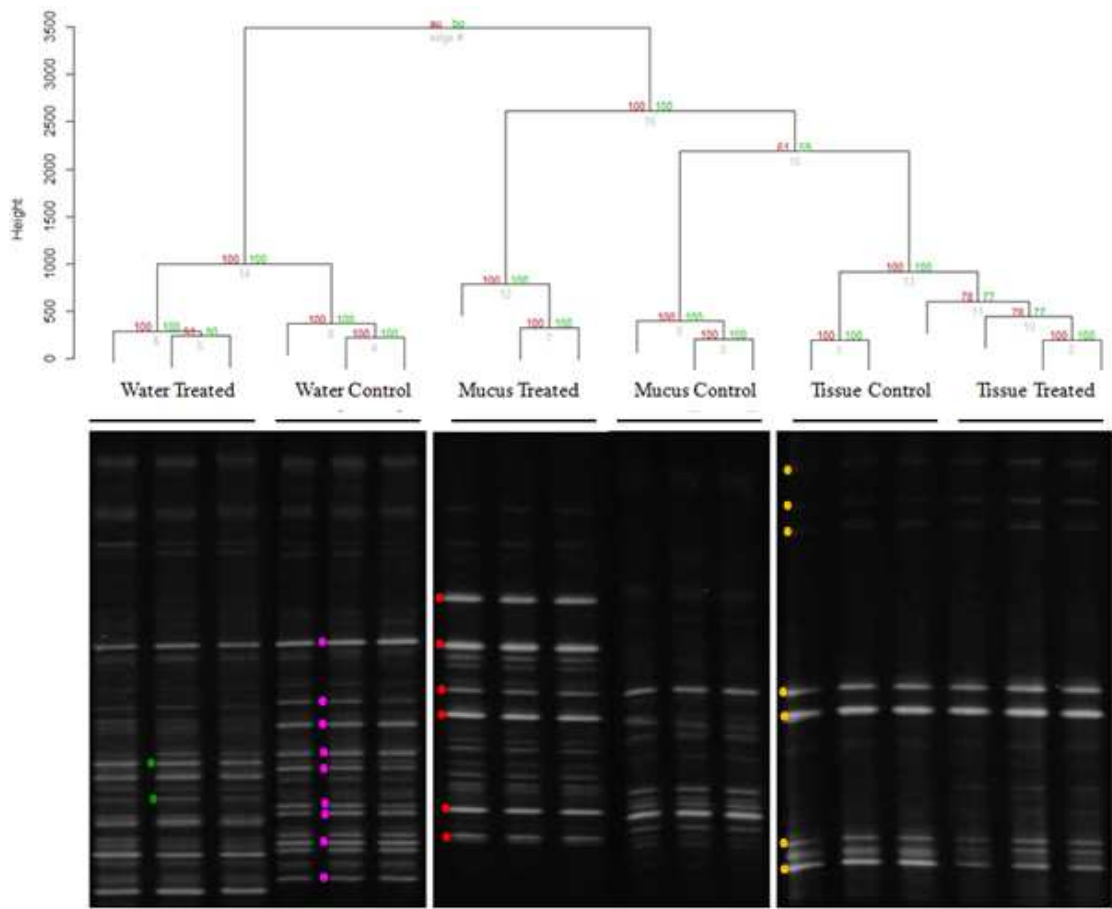**Figure 2.** *Bacterial abundance in mucus and tissue from corals exposed to elevated temperature relative to controls at ambient temperature. Bacterial abundance was determined by counting DAPI-stained samples under an epifluorescence microscope.*
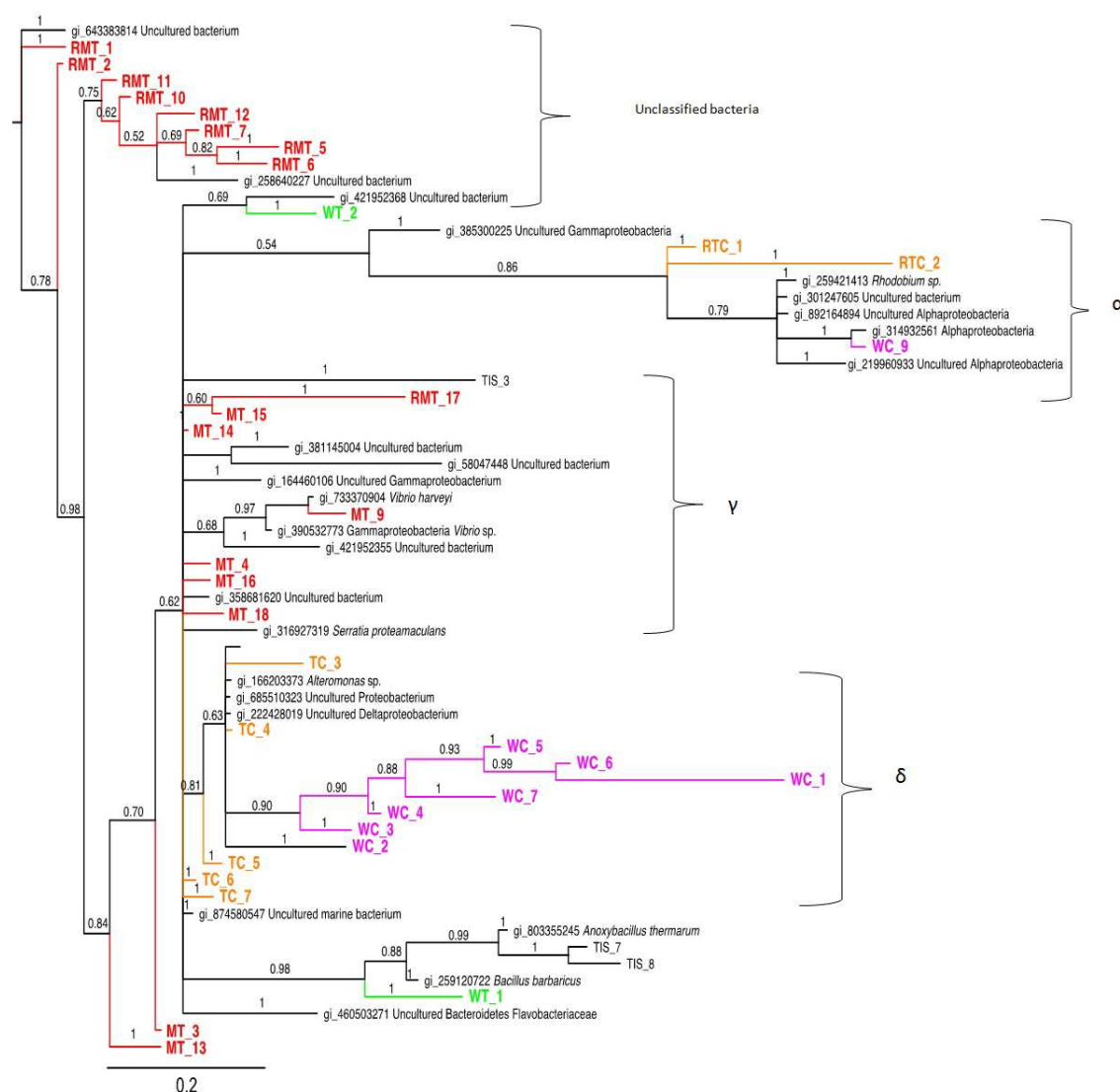
Figure 3. *Denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA V3 region in the coral tissue, mucus, and tank water analyzed after 10 days of thermal stress at 27±1°C versus 32±°C. Heirarchical clustering of DGGE profiles was implemented on pvclust. Approximately unbiased p-values are shown in red and bootstrap probabilities are in green. 2 Sequencing of distinct DGGE bands reveal the diverse affiliation of bacteria associated with the coral. Colors in (2) represent the bands marked with colored circles in (1).*
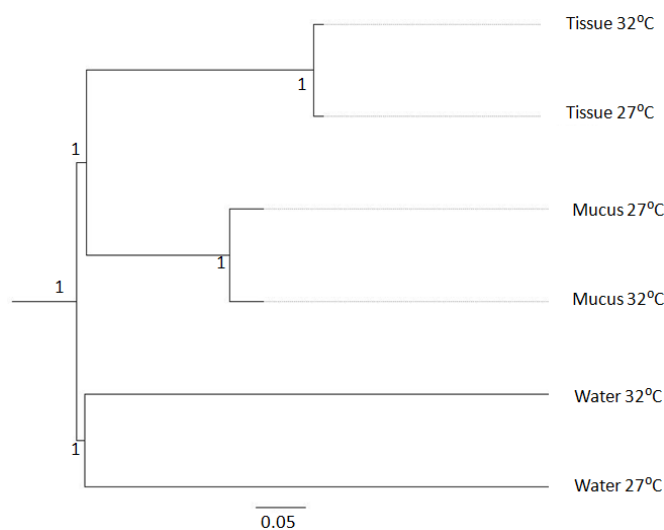

Figure 4. *Cluster Analysis of sequences obtain from microbial community 16s rRNA (v4 region) gene. Cluster analysis was performed by cluster environment wiht thetayc values in a Unifrac metric analysis in mothur. The bar represent a weighted theytayc distance of 0.05.*

3.3. Diversity of nifH Sequences

Clone libraries were prepared for the *nifH* gene sequences of mucus samples for both control and thermally stressed corals. Analysis of the *nifH* gene sequences obtained from the cloned isolates (n=150) showed diverse taxonomic

membership. Both the Shannon and Simpson indices vividly indicated that microbial diversity is lower in the control and treated mucus samples compared to mucus collected from corals *in situ* (Table 1).

Table 1. Number of OTU and diversity estimates for nifH sequences from mucus samples of A. digitifera。

Library	No. of clones analyzed	No. of OTU observed	ACE estimator	Chao estimator	Simpson index	Inverse simpson	Alpha	Shannon Diversity
27°C	50	32	48.85	48.8	0.7639	4.2353	2.626	1.5643
32°C	50	17	22.48	22.4	0.7975	4.9388	2.718	1.6880
In-situ	50	30	48.30	47.7	0.6844	3.1690	2.568	1.3379

Meanwhile, non-parametric Chao and ACE estimators revealed that the species richness in mucus decreased in samples exposed to high temperature (Table 1). The findings validate the initial observation that artificial setting triggers shift in bacterial diversity of corals. Unfortunately, rarefaction curves based on OTUs did not reach saturation for all the samples (Figure 5b) thus, leads to an incomplete coverage of the *nifH* gene libraries and the sequencing of additional clones abruptly increases the number of discovered OTUs.

### 3.4. Nitrogen Fixers in the Mucus Layer

In-situ mucus derived sequences were taxonomically diverse with nearly half of the sequence belongs to *Cyanobacteria* (Figure 5a). Among the class of bacteria, cluster 11 with 24 sequences, proves to be the most abundant demonstrating a 100% amino acid identity with an uncultured member of *Cyanobacteria* in the genus *Anabaena* (NZ_KB235896.1) and with similar identity with filamentous *cyanobacterium* (NZ_KB904821.1) (Table 2).

*Table 2. Abundant nitrogen fixing bacterial clusters associated with the Acropora digitifera mucus layer.*

Cluster	Class	Closest match in Blastn (% identity) Accession no.	No. of clones	Abundance (%) Mucus 27°C	Mucus 32°C	Mucus in situ
1	γ-Proteobacteria	Azotobacter (100) NC_012560.1	1	2	--	--
2	ε -Proteobacteria	Arcobacter(92) NC_017192.1	7	2	10	2
3	γ-Proteobacteria	Rahnella (82) NC_016819.1	2	--	4	--
		Tolumonas (84) NC_012691.1	3	--	--	6
		Azotobacter (90) NC_012560.1	4	8	--	--
		Teredinibacter(90) NC_012997.1	7	6	4	4
		Methylomonas(84) NC_015572.1	1	2	--	--
		Allochromatium(88) NC_013851.1	1	--	--	2
		Marinobacterium(99) NZ_AUAZ01000022.1	2	4	--	--
4	α -Proteobacteria	Methylocystis(88) NC_018485.1	1	2	--	--
		Bradyrhizobium (97) NC_017082.1	2	4	--	--
5	γ-Proteobacteria	Teredinibacter(89) NC_012997.1	6	4	8	--
		Azotobacter (91) NC_012560.1	12	8	16	--
		Pseudomonas (98) NC_009434.1	1	2	--	--
		Dickeya(85) NZ_CM001857.1	2	2	--	2
		Vibrio(87) NZ_BBJY01000001.1	1	--	--	2
		Tolumonas(88) NC_012691.1	1	--	--	2
6	δ-Proteobacteria	Desulfovibrio(89) NC_016803.1	8	6	8	2
		Desulfomicrobium(80)NZ_AUAR01000008.1	3	--	6	--
7	δ-Proteobacteria	Desulfobacter(84)NZ_JQKJ01000010.1	4	2	6	--
		Desulfobacterium(84) NC_012108.1	1	--	2	--
8	δ-Proteobacteria	Desulfomicrobium(80)NZ_AUAR01000008.1	1	--	2	--
		Desulfovibrio(75) NC_016803.1	1	--	2	--
9	γ-Proteobacteria	Azotobacter (83) NC_012560.1	1	--	2	--
10	γ-Proteobacteria	Teredinibacter(86) NC_012997.1	1	--	2	--
11	Cyanobacteria	Cyanothece(84) NC_011884.1	7	4	4	6
		Filamentous cyanobacterium (86)NZ_KB904821.1	7	--	--	14
		Microcoleus(84) NC_019738.1	1	--	--	2
		Leptolyngbya (83) NZ_KB731324.1	1	--	--	2
		Anabaena(100) NZ_KB235896.1	2	--	--	4
		Rivularia(82) NC_019678.1	1	--	--	2
		Calothrix(85) NZ_KB217478.1	1	2	--	--
		Stanieria(95) NC_019748.1	1	--	--	2
		Myxosarcina(89) NZ_JRFE01000060.1	3	--	--	6
12	δ-Proteobacteria	Desulfovibrio(78)NZ_AUBQ01000013.1	2	--	--	4

Cluster	Class	Closest match in Blastn (% identity) Accession no.	No. of clones	Abundance (%)		
				Mucus 27°C	Mucus 32°C	Mucus in situ
13	γ-Proteobacteria	Azotobacter(90) NC_012560.1	1	--	2	--
14	Cyanobacteria	Filamentous cyanobacterium (86)NZ_KB904821.1	1	--	--	2
15	δ-Proteobacteria	Desulfobacterium(84)NC_012108.1	1	--	2	--
		Delsulfobacter(78) NZ_CM001488.1	1	2	--	--
		Desulfospira NZ_ATUG01000002.1	2	--	--	4
16	γ-Proteobacteria	Azotobacter(88) NC_012560.1	1	--	--	2
17	β-Proteobacteria	Dechloromonas(94) NC_007298.1	1	--	--	2
		Burkholderia(78) NC_007952.1	1	--	--	2
18	γ-Proteobacteria	Halorhodospira(85) NC_008789.1	1	--	--	2
19	α -Proteobacteria	Bradyrhizobium (97) NC_017082.1	1	2	--	--
20	γ-Proteobacteria	Azotobacter (91) NC_012560.1	10	4	10	6
21	δ-Proteobacteria	Pelobacter(85) NC_007498.2	1	--	--	2
22	γ-Proteobacteria	Teredinibacter(88) NC_012997.1	1	--	2	--
23	γ-Proteobacteria	Azotobacter (86) NC_012560.1	1	2	--	--
24	γ-Proteobacteria	Methylomonas(84) NZ_BBCK01000057.1	1	--	--	2
25	δ-Proteobacteria	Desulfobacter(88)NZ_JQKJ01000010.1	4	--	6	2
26	Cyanobacteria	Filamentous cyanobacterium (86)NZ_KB904821.1	1	--	--	2
27	α -Proteobacteria	Methylocyctis(90) NC_018485.1	1	2	--	--
		Bradyrhizobium (97) NC_017082.1	2	4	--	--
28	α -Proteobacteria	Xanthobacter(85) NZ_JAFO01000001.1	1	2	--	--
29	α -Proteobacteria	Xanthobacter(89) NC_009720.1	1	2	--	--
30	β-Proteobacteria	Burkholderia(91) NC_007952.1	1	--	--	2
31	α -Proteobacteria	Confluentimicrobium(90) NZ_CP010869.1	1	2	--	--
32	Cyanobacteia	Anabaena(84) NZ_KB235896.1	3	--	--	6
33	Cyanobacteia	Cyanothece(82) NC_011729.1	1	--	--	2
34	γ-Proteobacteria	Halorhodospira(85) NC_008789.1	1	2	--	--
35	ε -Proteobacteria	Arcobacter(85) NC_017192.1	1	2	--	--
36	Bacteroidetes	Pedobacter(90) NZ_AWRU01000033.1	1	2	--	--
37	Cyanobacteria	Nostoc (83) NC_014248.1	1	--	2	--
38	Cyanobacteria	Stanieria(95) NC_019748.1	1	2	--	--
39	Unclassified bact.	Uncultured bacteria	1	2	--	--
40	Unclassified bact.	Uncultured bacteria	3	6	--	--
Total in 40 cluster			150	50	50	50

Analysis is based on 150 sequences. A cluster is defined as *nifH* gene clones that have more than 75% sequence identity. The consensus sequence of the cluster was used in searching the Genbank Database (NCBI database using Blastn). *nifH* was only amplified from the mucus fractions (See Materials and Method).
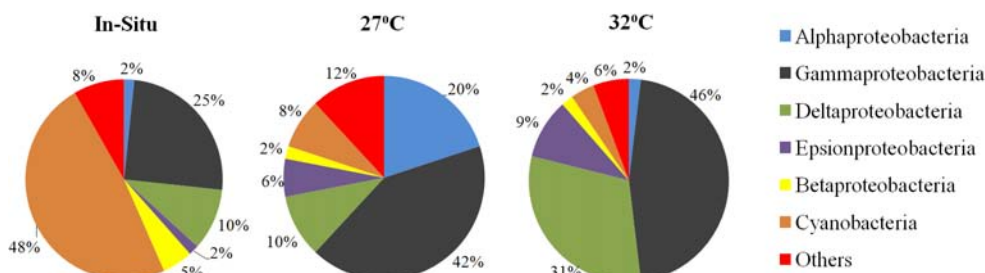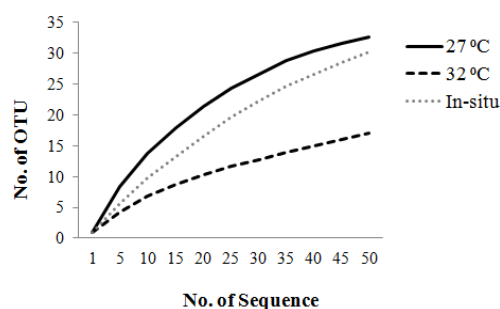
Figure 5. *Composition of nifH operational taxonomic units (OTU) from coral mucus. (A) Approximately 77-80% of mucus nifH OTUs belong to Proteobacteria while about 13% belong to Cyanobacteria. A lower number of nifH OTUs were obtained from all the samples. Based on the rarefaction curve (B), this is likely due to insufficient sample size.*

Similarity of sequence identity, roughly 95%, was also observed in close homology to genus *Staniera*. Interestingly, a number of particular taxa was observed in the in-situ samples only, like the group of *Aeromonadales* and *Oscillatorialles* (Figure 6), as well as *Betaproteobacteria*. These sequence are affiliated with the group of *Burkholderiales* and *Rhodocyclales,* a group of symbiotic diazotrophs found in the root nodules of legume plant.

Among the remaining mucus-derived *nifH* sequences retrieved, 40 sequences was taken from uncultured member of Gammaproteobacteria, a close homology to *Azotobacter* with a perfect identity of 100%, 8 sequences were found in the class of *Epsilonproteobacteria* and only one (1) belonged to the *Bacteroidetes* class.

Likewise, majority (77-80%) of mucus nifH sequences belong to Proteobacteria, while *Cyanobacteria* was with 13% (Figure 5a). A wide distribution of *Alphaproteobacteria* across control samples was detected with close homology to the group of *Rhizobiales* (*Xanthobacter, Methylocystis, Hyphomicrobium*) and *Rhodobacterales* (Figure 6).

Remarkably, a number of the bacterial taxa that were observed in-situ samples were not detected in control samples, especially the group of *Cyanobacria* in the order *Oscillatoriales*. Meanwhile, bacterial class of *Gammaproteobacteria, Epsilonproteobacteria* and *Deltaproteobacteria* (Figure 5a), increases its frequency when exposed in the hatchery set-up. The finding clearly suggest that (1) artificial setting in the hatchery does not favor the above mentioned bacterial taxa, (2) either, the shift of bacterial composition affect the holobiont metabolism, (3) or corals manipulate the microbiota to adapt in the hatchery settings. Hence the increase of other bacterial taxa is favored for holobiont survival.

3.5. Effect of Thermal Stress on the Nitrogen Fixing Bacterial Community

Diazotrophic association in the treated (32°C) mucus samples revealed a clear pattern of shift in bacterial communities. A spike increase in frequency was detected in the class *Gammaproteobacteria* specifically among *Alteromonadales* and *Pseudomonadales* group (Figure 6). Also, an increase was evident in the group of *Epsilonproteobacteria* which is closely similar to *Campylobacterales*. Furthermore, the same pattern was also observe in the group of *Desulfovibrionales and Desulfobacterales* (Figure 6) otherwise known as sulfate reducers.

On the other hand, the class of *Cyanobacteria* and *Alphaproteobacteria, nifH* sequences decreases in treated mucus samples relative to the control and In-situ samples (Figure 5a and 6). The findings confirms that elevated water temperature greatly affect the diazotrophic community and the coral survival.

4. Discussion

Corals take the majority of carbon requirement from their symbiotic associations with zooxanthellae [41, 59, 64, 65, 66,

88]. Typically, corals are passive suspension feeders which usually trap particles and bacteria in its mucus as a source of nutrients [21, 42, 71]. Besides being the direct source of nutrients to corals through bacterivory, a number of studies cite new evidences that microbial members of the coral holobiont potentially contribute fixed nitrogen to either the coral polyp or the zooxanthellae [47, 67].
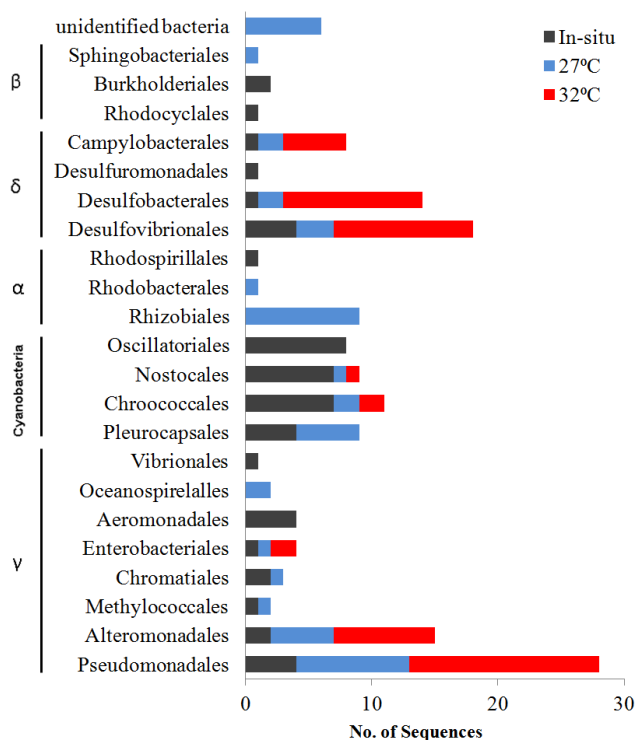

Figure 6. *Bacterial taxa of associated nifH sequences from the coral mucus samples reveals shift in composition as expose to different environmental conditions. Control (27°C), Treated (32°C) and In-stu. Symbols represents each proteobacteria taxa; (γ) gamaproteobacteria; (α) alphaproteobacteria; (δ) deltaproteobacteria; (β) betaproteobacteria.*

Lesser et al. [44], Ueda et al. [79] and Lema et al. [45, 46], reported that a large number of nitrogen fixing bacteria occurs in the coral mucus and tissue layer of *Montastrea cavernosa*. For instance, *V. harveyi* and *V. alginolyticus* both are capable of nitrogen fixation in coral mucus and dominate the culturable nitrogen fixing bacteria of the Brazilian coral *Mussismilia hispida* [10, 11]. Also, a comprehensive survey of nitrogen fixing bacteria recovered from mucus of three corals in Great Barrier Reef disclosed that the diversity of the *nifH* in mucus was generally similar to that of the surrounding seawater [81]. All these studies shows that nitrogen fixing bacteria is one of the component of the host microbiota to shape physiology of the coral when under stress circumstances.

The libraries derived from mucus samples of *A. digitifera* species during 10th day thermal stress treatment reveals that diazotrophic communities are characterized by high diversity. Majority of the *nifH* sequences (77%) retrieved from coral mucus belongs to *Gammaproteobacteria* class. *Gammaproteobacteria* groups is closely affiliated with

Azotobacter and *Teredinibacter*. The former occurs as an intracellular endosymbiont in the gills of marine bivalves which provide host with enzyme including cellulases and nitrogenase critical for nitrogen deficiency [2, 18, 44, 84, 85]. While the latter, *Azotobacter*, is a free living diazotrophic bacteria with metabolic capabilities including atmospheric nitrogen fixation by conversion to ammonia and have a unique system of distinct nitrogenase enzyme [2, 35, 36, 37, 44, 54].

Obviously, bacteria plays a vital role in every ecosystem, enabling nitrogen to be made available for all organism. The ability to fix nitrogen is an important phenotypic trait of diazotrophic bacteria, and *nifH* has been use to distinguish representative at the clade level [2, 15, 19]. The findings of the study reveals that the domineering type of bacteria in the mucus of *A. digitifera* coral species is that of *Gammaproteobacteria class*. Much, the study indicates that ammonium is potentially abundant in coral mucus.

Meanwhile, majority of *nifH* sequences spotted were affiliated with the group of *Cyanobacteria*, regarded as main drivers of nitrogen fixation in corals [20, 44, 65]. Sequences retrieved were closely associated with *Chroococales*, *Nostocales* and *Oscillatoriales*. Cyanobacteria plays an essential role in modern coral reefs ecosystem being the major component of epiphytic, epilithic, and endolithic communities and of microbial mats as well [26, 50]. In the microbial mats of reefs, *Filamentous* cyanobacterium strikingly dominates the ecosystem [1, 2, 4, 5, 8]. The diversity of cyanobacterial mats inhabiting different environments have been the focus of numerous studies on the several places such as: Tikehau atoll-French Polynasia [1, 66], in New Caledonia [8, 9], and in the Western Indian Ocean in Zanzibar-Tanzania [4]. These studies identified different structures which differs in appearance [56], species composition, mode of growth and affiliation to the benthic organism including the coral and their substrate. Thus, these types of bacteria are vital during calcification and decalcification process [8, 9], much, in the stability of coral-algae symbosis [32, 33, 63, 76, 77].

A large number of *nifH* genes are closely related to sulfate reducers, including *Desulfovibrionales* and *Desulfobacterales* representing the second largest group retrieved from the mucus samples. Kimes and colleagues [37] determined that 22% of *dsr* genes in corals belong to the *Deltaproteobacteria* subclass and indicated that inorganic sulfate is a potential source of sulfur for the corals holobiont. In a study that investigate *nifH* in corals, *nifH* sequences close to *Desulfovibrio* and other uncultured sulfate reducers were found [61]. And validating the findings of Olson and Colleagues [61], the study discovered *nifH* sequences affiliated with *Desulfovibrio* in the clone libraries for *A. digitifera*.

Interestingly, *Alphatroteobacteria* phylotypes which were closely related to bacterial species belonging to the *Rhizobiales* order were found in all mucus samples. *Rhizobia* are soil bacteria that inhabited nodules of legume plants roots [28, 45, 46]. *Also, Rhizobia* fix nitrogen, enabling plants to thrive and reproduce in nitrogen poor environments and in return of carbon and amino acid [22, 23, 43, 45, 46, 51].

In the desire to highlight the importance of *Rhizobia* group of bacteria, Rhizobium-affiliated sequences were clustered and was closely related to *Bradyrhizobium,* one of the most commonly occurring rhizobia that forms symbioses in the nodules of legumes plants. The study of Lema et al [45, 46] found a vertical transfer of diazotrophs from parental colonies of the *Acropora millipora* to their larvae, mostly an *Alphaproteobacteria* of the group *Rhizobiales*. The vertical transfer of diazotrophs further suggest a beneficial role of the group for holobiont functioning.

Furthermore, a number of *nifH* sequences were detected to be affiliated with *Xanthobacter*, a nutritionally versatile bacterium, with an unprecedented array of metabolic capability relevant in nitrogen fixation [52, 54]. Although the magnitude of transfer of fix nitrogen from diazotrophs into other compartments of the coral holobiont (e.g *Symbiodinium*) has not been quantified, recent studies shows that nitrogen fixation is a highly energy consuming process which requires 16 mol of ATP for the reduction of 1 mol of dinitrogen [54, 65]. Therefore, nitrogen fixation is energetically more costly than other mechanism of ammonium assimilation leading to the preference of other source of fixed nitrogen if available. Hence, nitrogen fixation serves as mechanism to counter act shortage of environmental nitrogen availability, and maintain a constant nitrogen supply for symbiotic based primary production in corals. These views is further supported by the findings of Olson [61] and Lesser [44] who reported a positive correlation of diazotrophs abundance with density and DNA content of *Symbiodinium* cells.

Despite the overall comparatively small contribution to the nitrogen budget of the coral holobiont, nitrogen fixation remains essential to the stability of the coral-algae symbiosis. If the ability of these marine diazotrophic bacteria to fix nitrogen in the association with the coral host is demonstrated experimentally, this will propel a deeper understanding of the evolution of nitrogen fixation and holobiont ecology. Consequently, it will constitute an important functional link between carbon and nitrogen fixation within the holobiont, and thus, contribute to the survival of corals in a highly oligotrophic reef environment.

Clearly, there is still much to learn about the diversity of nitrogen-fixing prokaryotes and their role in coral microbial communities, but our data suggest that nitrogen fixers associate with *A. digitifera* corals were diverse and may provide insight into the functional role of bacteria in support of the stress response of corals.

5. Conclusion

Taken together, the data indicated that nitrogen fixing bacteria exist in coral mucus and this layer paves the way to a diverse diazotrophic community. The specificity of mucus diazotrophs to each of the coral species provides support for the holobiont model of coral symbioses. Also, the overall consistency in the identification of diazotrophic *nifH* sequences associated with *A. digitifera* and from other corals

suggest that these microbial groups is essential in nitrogen cycle within the coral holobiont. More importantly, changes in diazotrophic communities directly reflect shifts in environmental parameters, such as increase seawater temperature, and could be used to detect changes in coral fitness in response to environmental change.

Conflict of Interest

Acknowledgements

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