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Microbial Diversity and Transcriptome Profiling in Coral Holobionts

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by

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DEDICATION

To my parents, Kayoko and Akio, who sent me on this trip called life.
And to my wife, Kathrin, my inexhaustible source of energy.

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

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Coral reefs are often considered as the proverbial canary in the mine for the oceans. Their current decline alarmingly correlates with indirect and direct anthropogenic stress resulting in increased frequencies and geographic spread of mass mortality, i.e., "coral bleaching", and disease events. Thus, a rapid advancement in the understanding of the factors determining coral health and disease is of great importance. The post-genomic era has propelled both the development of high-throughput technologies and a shift in biological research from reductionist to system-level approaches. By viewing an organism as an "integrated and interacting network of genes, proteins, and biochemical reactions", the nascent field of systems biology seeks to understand biological processes at a holistic level.

In this dissertation, corals are considered as superorganisms, or "holobionts", i.e., biological entities composed of a host organism and all of its associated microorganisms. The microbial component has been shown to play key roles in the functioning of the coral holobiont, but much remains to be studied about its 1) diversity, 2) response to stress, and 3) influence on the physiology, ecology, and evolution of the host. To address these gaps, I have applied a combinatorial approach of 454-sequencing, microbial community profiling (Phylochip), algal and host genotyping, as well as cDNA microarrays. The results include the most comprehensive census of coral-associated bacteria so far and illustrate that corals provide specialized habitats for an extremely diverse consortium of bacteria, including taxa that are often unknown, as well as rarely or not detected in the seawater. In diseased corals, bacterial communities profiles shifted and displayed

a higher abundance of bacteria that are commonly found in other diseased marine invertebrates. Finally, correlating algal genotype and coral host transcriptomic data revealed a substantial interaction between microbial symbionts and host gene expression.

These results represent initial efforts towards capturing the parts lists, i.e., the microbial diversity in coral holobionts, and integrating them with host transcriptomic data. The relevance of the results are discussed in the context of an envisioned coral (eco)systems biology approach to advance our understanding of coral health and disease.

1 General Introduction

1.1 Ecological and economic importance of coral reefs

Coral reefs are the most biodiverse, ecologically mature and structurally complex marine ecosystems. Their global distribution extends from approximately 30° N to 30° S of the equator and is limited to regions where water temperatures exceed about 18 °C. Corals also depend on the availability of light, which confines the habitable areas conducive for growth and reproduction to the photic zone of the water column (approx. < 50 m depth). Furthermore, although coral reefs exist in environments that are low in nutrients, they support an extremely diverse profusion of life; a phenomenon that has also become known as Darwin's paradox (Darwin, 1842).

In coral reefs ecosystems, net primary production can be up to three orders of magnitude greater than in open ocean waters (Hatcher, 1988). This high productivity sustains services that are important not only for the reef system itself, but also for adjacent ecosystems of the tropical seascape to which they are functionally linked. For example, mangrove forests and seagrass beds represent important nurseries for juvenile fish and other organisms that return to coral reefs once they reach adult life stages (Ogden and Gladfelter, 1983).

With an estimated value of US\$ 375 billion per year (Costanza et al., 1997), coral reefs generate crucial sources of income for hundreds of millions of people, mainly through their roles in tourism, fisheries, and the discovery of bioactive substances (e.g., Carte, 1996). Tourism represents a major income source for many countries associated with coral reefs. In 1990, about US\$ 9 billion were generated by tourism in the Caribbean region; coral reefs of Florida alone have been estimated to generate about US\$ 1.6 billion annually (Jameson et al., 1995). Food acquired through fisheries is a main nutritional source for large coastal communities in developing countries. Coral reefs provide about 20 - 25 % of the total fish catch in those countries. Many Pacific islands obtain as much as 90 % of the animal protein consumed from marine sources (Jameson et al., 1995). Coral reef organisms are also recognized for their potential to produce bioactive substances (Munro et al., 1999). Global cancer research is focusing on extracts from marine organisms (Blunt et al., 2007) and the potential of using coral skeletons as bone grafts has been investigated

since the 1970s (Wu et al., 2009). Coral reefs are also important for the physical protection of subtropical and tropical coastlines from erosion and flooding. This often underestimated protective service has been estimated as a value of US\$ 40,000 million $\text{km}^{-1} \text{yr}^{-1}$ on a coastline with major infrastructure (Cesar, 1996).

1.1.1 Status of coral reef ecosystems

The United Nations declared the year 2010 as the "International Year of Biodiversity" in an effort to combat the current rates of biodiversity loss. A recent assessment suggests that worldwide about one-third of reef-building corals are at elevated risk of extinction (Carpenter et al., 2008) with the Caribbean basin being one of the most heavily impacted regions (Gardner et al., 2003). Since 1950, an estimated 19 % of coral reefs have been effectively destroyed, 15 % are in a critical state, and 20 % are threatened to be lost within the next two to four decades (Wilkinson, 2008). Given the ecological and economic importance of coral reef ecosystems, a rapid advancement in understanding the factors determining coral health and disease is a pressing task.

1.2 The coral holobiont as a biological entity

Coral reefs provide a complex structural framework as a result of calcium carbonate deposition by reef-building corals (Cnidaria:Hexacorallia:Scleractinia). Variations in biological (e.g., species diversity and colony morphology) and environmental factors (e.g., the availability of light, gradients of nutrient concentrations, and water flow) contribute to niche diversification, which in turn promotes the enormous diversity of macroorganisms typically observed on coral reefs (Figure 1.1A; reviewed in Ainsworth et al., 2009). Similarly, each coral colony provides a number of microhabitats for microorganisms. On a broader scale, there are three distinct habitats in the coral colony that can be populated by microbes: the surface mucus layer, the coral tissue and the underlying calcium carbonate skeleton (Figure 1.1C; reviewed in Rosenberg et al., 2007). The surface mucus layer separates the seawater environment from the coral tissue layer (the coral animal), which in turn covers the coral skeleton. Each of these layers provides suitable niches for colonization by a diverse array of microorganisms, including protists, fungi, bacteria, and archaea, as well as viruses. Thus, corals, like other animals and plants, can be considered as superorganisms or "holobionts" (Rohwer et al., 2002), i.e., as functional entities composed of the host organism and a complex, interacting network of microbial associates. Although the role of other microorganisms has been discussed in the literature, the following subsections will only focus on the endosymbiotic, algal symbionts of corals and coral-associated prokaryotes (bacteria and archaea).

1.2.1 *Symbiodinium* spp.

Reef-building corals maintain a well-studied, mutualistic partnership with dinoflagellate algae in the genus *Symbiodinium*. As endosymbionts they are found inside the cytoplasm of endo- and gastrodermal cells of corals within vacuoles (Trench, 1979) that are now commonly referred to as "symbiosomes". They translocate up to 95 % of their photosynthates such as glycerol, glucose, and amino acids to the coral host (Muscatine, 1990). In turn, *Symbiodinium* cells efficiently recycle the metabolic waste products of their hosts, while residing in an environment well-protected from herbivorous predators (Hoegh-Guldberg, 1999).

Until the late 1970s, *Symbiodinium* sp. had been considered a single ubiquitous species named *Symbiodinium microadriaticum* (Freudenthal, 1962). This monotypic status changed with emerging biochemical and morphological evidence at that time (Trench, 1979; Schoenberg and Trench, 1980a,b). In the early 1990s, molecular genotyping tools revealed that *Symbiodinium* is a diverse genus whose member species are now broadly grouped into eight different clades (reviewed in Coffroth and Santos, 2005). While some coral species seem to associate with only one particular clade of *Symbiodinium*, other coral species (e.g., *Montastraea faveolata*) are known to establish associations with multiple *Symbiodinium* clades (e.g., Rowan and Knowlton, 1995). Studies investigating the latter group have revealed that different combinations of coral-*Symbiodinium* partnerships can affect important properties of the coral holobiont, including : 1) adaptation to different environmental conditions (Rowan and Knowlton, 1995; Iglesias-Prieto et al., 2004), 2) growth, carbon fixation, and photosynthate transfer rates (Little et al., 2004; Loram et al., 2007; Stat et al., 2008; Cantin et al., 2009; Mieog et al., 2009), and 3) susceptibility to thermal stress (Rowan et al., 1997; Baker et al., 2004; Middlebrook et al., 2008; Sampayo et al., 2008; Mieog et al., 2009). Despite these apparent functional variations in different host-*Symbiodinium* combinations, the effect of different *Symbiodinium* genotypes on global coral gene expression, i.e. the transcriptome, has not yet been examined. This, however, is an important step to elucidate the underlying molecular mechanisms that contribute to phenotypic plasticity and environmental adaptation in corals.

1.2.2 Bacteria and archaea

In 1676, Antonie van Leeuwenhoek observed, for the first time in history, bacteria (which he named: "animalcules") using a self-designed microscope (Porter, 1976). Some three centuries later, it was another technological revolution that enabled Carl Woese to set a further milestone in contemporary biology: the sequencing of the small-subunit ribosomal RNA gene from organisms representing the three domains of life: Bacteria, Archaea, and Eucarya (Woese and Fox, 1977; Woese et al., 1990).

Early attempts to quantify the abundance of bacteria in a sample (e.g.,

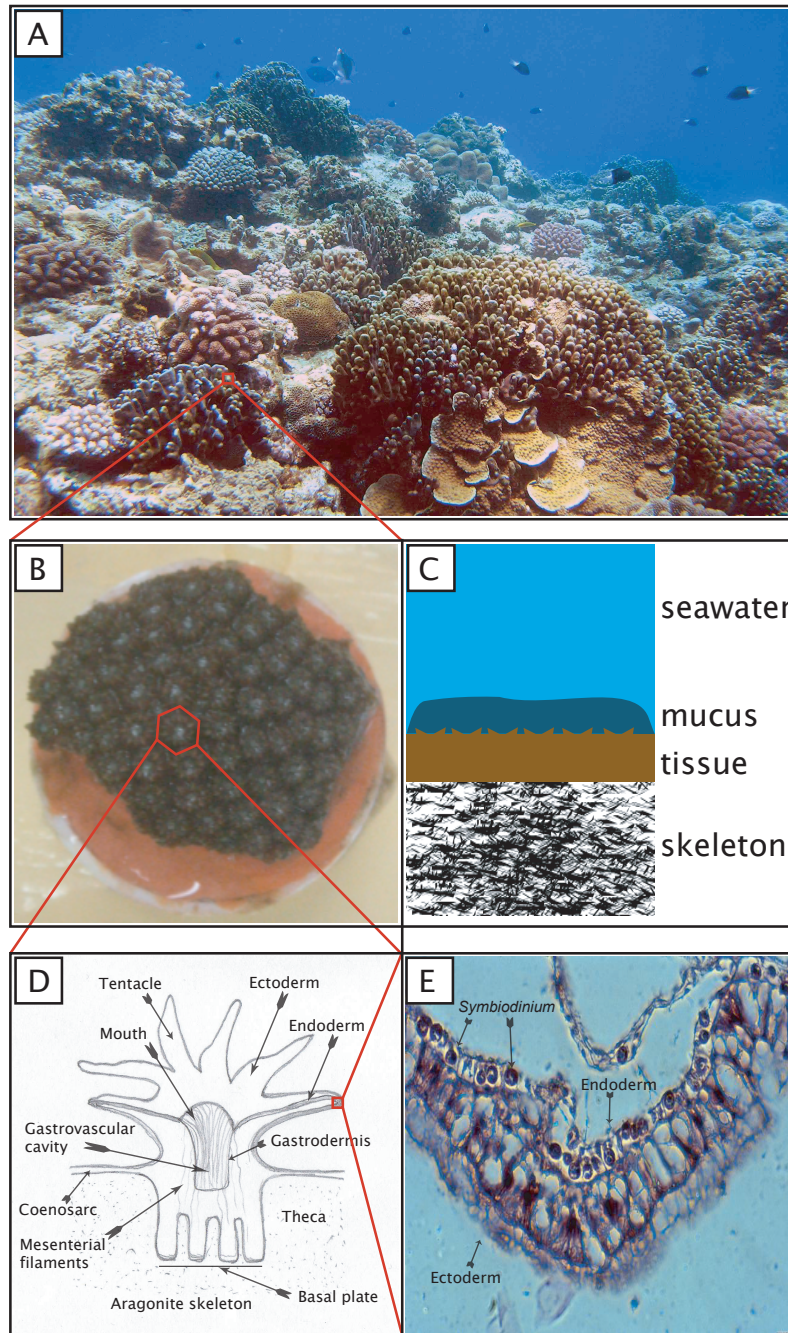


Figure 1.1: Ecological niches in coral reef ecosystems at different scales. (A) Coral reef displaying high biodiversity of macroorganisms (Kuroshima Reef, Okinawa, Japan). (B) *Montastraea faveolata* colony fragment collected at: La Bocana Reef, Puerto Morelos, Mexico. (C) Schematic representation of seawater, coral surface mucus, tissue, and skeleton as distinct microbial habitats. (D) Anatomic features of a coral polyp. (E) Histological preparation of coral tissue showing *Symbiodinium* cells residing inside endodermal cells (image courtesy of Dr. Scott Santos).

seawater) were based on the presumption that each colony that grew on a nutrient agar plate was derived from a single bacterial cell. In the late 1970s and early 1980s, improvements in staining techniques and advancements in epifluorescence microscopy methods revealed that bacterial abundances in seawater samples were orders of magnitude greater than previously thought; a discrepancy that was later dubbed "the great plate count anomaly" (Staley and Konopka, 1985). The measurement and interpretation of microbial diversity (i.e., the number of different taxa) had been impeded by the inability to cultivate the great majority of microbes (possibly >99 %) in a given sample (Fuhrman and Campbell, 1998). Pace et al. (1986) demonstrated that one could overcome this limitation by sequencing 16S ribosomal RNA (16S rRNA) genes that were directly PCR-amplified from environmental DNA extracts (i.e., cultivation-independent) using universal primers targeting conserved regions of the 16S rRNA gene. A similar, less costly approach to investigate complex microbial populations in environmental samples was introduced by Muyzer et al. (1993), which is based on the separation of 16S rRNA amplicons by denaturing gradient gel electrophoresis (DGGE).

Initial efforts to analyze the microbial composition in corals using a cultivation independent approach have revealed highly diverse bacterial and archaeal taxa (Rohwer et al., 2001, 2002; Wegley et al., 2004; Kellogg, 2004). Rohwer et al. (2002) reported 430 distinct ribotypes in 14 samples from three different coral species (*Montastraea franksi*, *Diploria strigosa*, *Porites astreoides*) of which ~50 % were less than 93 % similar to previously published sequences. Interestingly, the authors showed that these coral species harbored distinct bacterial communities, irrespective of temporal (1 year separation) or spatial (Panama vs. Bermuda) separation of sample collection. Other studies have suggested that seawater, coral surface mucus layer, coral tissue, and coral skeleton (Figure 1.1C) represent habitats with distinct microbial communities (reviewed in Rosenberg et al., 2007).

The function of coral-associated bacteria has been hypothesized to involve ecological, physiological, and possibly adaptive roles (Zilber-Rosenberg and Rosenberg, 2008) in the coral holobiont. For example, Ritchie (2006) demonstrated that mucus from healthy *Acropora palmata* colonies displayed antibacterial properties including against known pathogenic strains. Cyanobacterial endosymbionts have been attributed to the fixation of nitrogen, which potentially is a limiting element in the coral holobiont (Williams et al., 1987; Shashar et al., 1994; Lesser et al., 2004). During times of coral bleaching, i.e., when corals lose their *Symbiodinium* populations, endolithic cyanobacteria may also serve as an additional source of energy (Fine and Loya, 2002). More recently, it has been proposed that the hologenome, that is, the sum of the genetic information of any animal host and its associated microbiota should be regarded as one unit of selection (Zilber-Rosenberg and Rosenberg, 2008). It is argued that changes in the diverse microbial community (i.e., the gene pool of the hologenome) could occur rapidly (i.e., at ecological time scales) and potentially result in an advantage for particular coral holobionts to survive environmental change (i.e., at evolutionary time

scales). While these hypotheses remain to be tested, it is clear that such an endeavor necessitates the ability to comprehensively characterize the microbiota of an organism at different levels of variability both spatial and temporal, as well as environmental disturbances.

1.2.3 Coral bleaching and coral diseases

The disruption of the symbiotic relationship between corals and *Symbiodinium* is commonly known as coral "bleaching" due to the pale appearance of coral tissues when photosynthetic pigments and/or algal cells are lost. Bleaching can be caused by a number of abiotic and biotic factors (reviewed in Douglas, 2003). However, mass bleaching events 1) are strongly correlated with abnormally high seawater temperatures (Hoegh-Guldberg, 1999), 2) have devastated coral reefs worldwide (Wilkinson, 2008), and 3) are projected to increase in both frequency and geographic extent (Donner, 2009). Interestingly, it has been shown that elevated temperatures could also trigger a bacterial infection and subsequently induce coral bleaching (Kushmaro et al., 1996). In these cases, corals are proposed to suffer from a temperature-induced (> 25 °C) exposure to virulence factors (e.g., a toxin that inhibits photosynthesis and the antioxidant enzyme superoxide dismutase) produced by certain bacterial strains of the genus *Vibrio* (Rosenberg and Falkovitz, 2004). Despite this evidence, the role of bacteria as a cause of coral bleaching has been recently debated (Ainsworth et al., 2008; Rosenberg et al., 2009).

In addition to the threat of coral bleaching, entire coral populations are also afflicted by disease episodes, which have increased in the number of species affected and the geographical extent of their occurrences (Sutherland et al., 2004). More than twenty coral diseases have been described so far including: Yellow Blotch Disease, White Plague Disease, White Pox Disease, and Black Band Disease (Weil et al., 2006). In a few examples, it has been shown that the bacterial composition of diseased corals is different from healthy ones (Frias-Lopez et al., 2002; Pantos et al., 2003; Pantos and Bythell, 2006). Nevertheless, in only a few instances has the disease-causing pathogen been corroborated by Koch's postulates (reviewed in Rosenberg et al., 2007; Bourne et al., 2009). Likewise, little is known about the environmental drivers of coral diseases, although thermal stress and coral density appear to be positively correlated with disease outbreaks (Bruno et al., 2007). Finally, the nature of disease-causation has been the subject of recent debate, i.e., whether diseases are primarily caused by pathogens that may be present in a latent, non-virulent form, and become pathogenic when triggered by environmental disturbance (opportunistic pathogens) and/or by primary pathogens (Lesser et al., 2007; Work et al., 2008).

1.3 New tools to address current limitations

The late 1990s and early 2000s have witnessed a revolution in the power of nucleic acid sequencing through the development of "high-throughput" technologies. These technologies have been instrumental in the sequencing of entire genomes including the human genome (Lander et al., 2001; Venter et al., 2001), and launched the post-genomic era. Post-genomic research areas include (but are not restricted to): studying global patterns of gene expression (transcriptomics), protein abundance (proteomics), small-molecule metabolite profiles (metabolomics), and the integration of the data generated by these studies (systems biology).

The availability of new sequence information has also propelled the development of hybridization-based, high-throughput technologies such as (c)DNA microarrays and 16S rRNA gene microarrays (e.g., Schena et al., 1995; Wilson et al., 2002). The ability to simultaneously assay thousands of genes or 16S rRNA genes (16S rDNA) respectively, results in high data-to-cost ratios, and thus, allows for a far more comprehensive profiling of samples than comparable single-gene approaches (e.g., northern blots, quantitative real-time PCR, or 16S rDNA clone library sequencing).

1.3.1 Diversity and community shifts in coral-associated bacteria

Microbial diversity estimates in corals have so far been based on capillary or "Sanger" sequencing of 16S rDNA clone libraries with a sampling effort of typically < 100 sequences per sample (e.g., Frias-Lopez et al., 2002; Rohwer et al., 2002; Bourne and Munn, 2005; Pantos and Bythell, 2006; Barneah et al., 2007). Although taxonomic diversity estimates do not require high coverage of the sampled community, the direct identification of only 430 (while predicting 6000) bacterial taxa (Rohwer et al., 2002) clearly illustrates the difficulties associated with characterizing the highly diverse microbiota of corals. Mainly, the high costs associated with the construction and sequencing of 16S rDNA clone libraries poses a major obstacle in both obtaining more accurate diversity estimates of and profiling changes in coral-associated microbial communities.

Pyrosequencing, i.e., the massively-parallel sequencing of short segments of the 16S rRNA gene, also called "phylotyping", represents an attractive alternative to traditional Sanger sequencing-based approaches to study bacterial diversity. In 2006, Sogin et al. demonstrated the sequencing the hypervariable region 6 (V6) of the bacterial 16S rRNA gene to be a powerful method to investigate the taxonomic diversity in seawater samples. If the same technology were applied to corals, the sampling effort would increase by more than two orders of magnitude when compared to previous studies (see above).

The major strength of both sequencing-based technologies, i.e., 16S rDNA clone library sequencing and massively-parallel phylotyping of culture-independent

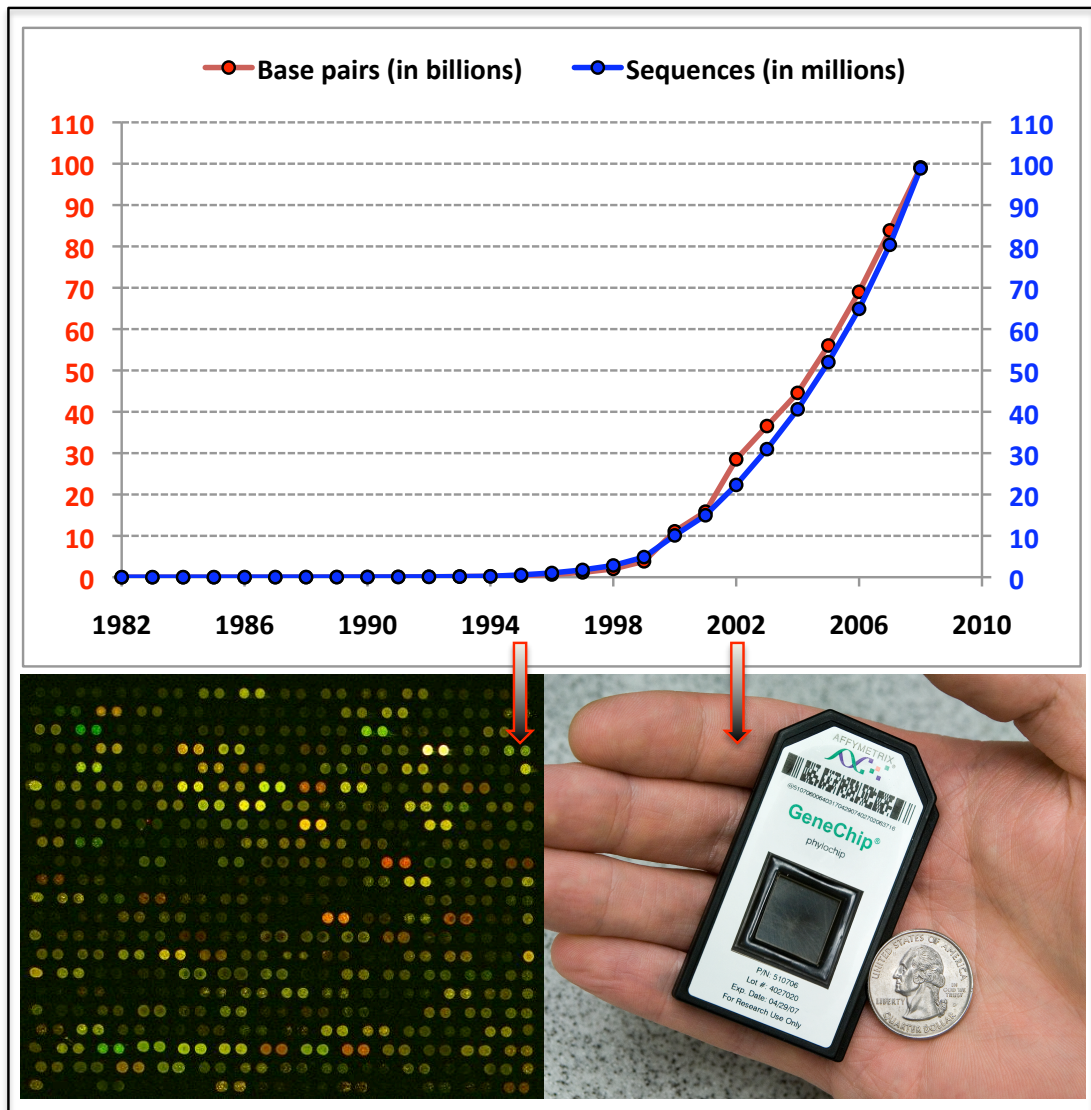


Figure 1.2: Growth of the National Center for Biotechnology Information’s Genetic Sequence Data Bank’s nucleotide sequence database and development of new technologies. The number of total base pairs and sequence entries has grown exponentially since the late 1990s (top). The availability of novel nucleotide sequences has spurred the development of innovative high-throughput tools, such as cDNA microarrays and 16S rRNA gene microarrays. Bottom, left: close-up of a cDNA microarray image showing a block of spot features with false-color signal intensities. Each spot represents a gene that is assayed for relative transcript abundances in competitively hybridized, fluorescence-labeled cDNA samples. Bottom, right: 16s rRNA gene microarray based on the Affymetrix GeneChip technology.

samples is the possibility to directly detect bacterial taxa by *de novo* sequence generation. However, the associated costs of these methods still represent a major disadvantage when compared to alternative, hybridization-based methods (DeSantis et al., 2007). The principle of the PhyloChip, a 16S rRNA gene microarray, is based on the hybridization of fragmented, fluorescence-labeled 16S rDNA mixtures to an array of immobilized detection probes. Thus, this hybridization-based technology has the potential to assay all 16S rDNA molecules present in a sample. The applicability of the PhyloChip and its advantage over 16S rDNA clone library sequencing has been demonstrated in a number of recent studies (e.g., DeSantis et al., 2007; Brodie et al., 2006, 2007). Due to the more cost-effective nature of this technology, it is possible to test for statistically significant differences between replicated groups of samples.

1.3.2 Gene expression profiling in corals using cDNA microarrays

Gene-expression microarrays represent a powerful tool to assay the gene-expression levels of thousands of genes in a single experiment. In 1995, Schena et al. (1995) published their work on the simultaneous assaying of 45 *Arabidopsis thaliana* genes, which hallmarked the use of miniaturized microarrays of complementary DNA (cDNA) to study gene expression in a high-throughput fashion. The automation of DNA sequencing technologies has spurred the development of cDNA microarrays for a multitude of organisms including: yeast, *E. coli*, human, mouse, as well as corals (Schwarz et al., 2008). In symbiotic corals or anemones, cDNA microarrays have thus far been used to study gene expression differences: 1) in symbiotic vs. aposymbiotic anemones (Rodriguez-Lanetty et al., 2006), 2) under thermal and UV stress (DeSalvo et al., 2008; Richier et al., 2008; Voolstra et al., 2009a), 3) during acclimatization of different source populations to different environmental conditions (Bay et al., 2009b), 4) within and between colonies (Bay et al., 2009a), 5) during the onset of symbiosis (Voolstra et al., 2009b), and 6) across distinct developmental stages (Grasso et al., 2008; Reyes-Bermudez et al., 2009).

1.4 Thesis objectives and chapter summaries

Over the past two decades, the advent of high-throughput technologies has caused a change in modern biology from reductionist approaches to global investigations of increasingly more complex systems. These systems range in scale from single molecules over whole cells and organisms to entire ecosystems. Given the current decline of coral ecosystems, it is timely to consider coral holobionts as biological systems and introduce novel tools to capture the systems parts inventory, study the parts dynamics in response to disturbance, and identify interactions be-

tween the parts. The following subsections summarize how such an approach was taken and novel tools used or introduced to study contemporary questions in coral biology.

The dissertation thesis presented here investigates the effects of environmental disturbances (disease and high temperature stress) on coral holobionts with an emphasis on the role of using modern high-throughput technologies.

1.4.1 Corals as habitats for diverse and underexplored communities of bacteria

The main objective of the work described in chapter 2 was to determine the taxonomic diversity and community structure of bacteria associated with phylogenetically diverse, healthy corals. I introduced massively-parallel phylotyping as a novel tool to the field of coral microbiology with the motivation to overcome current limitations (costs, resolution) in the comprehensive characterization of the bacterial component of the coral holobiont. Besides this technical and descriptive aspect of this goal, I also tested the hypotheses that:

- 1) the similarities of bacterial communities correlate with the known phylogenetic relationships of their hosts, and that
- 2) corals harbor bacterial communities unique in each coral species and distinct from the surrounding seawater.

The results of this study appeared as a manuscript entitled: "Threatened corals provide underexplored microbial habitats", published in PLoS ONE on March 5, 2010. A reprint of this publication is included as Chapter 2 of this thesis.

1.4.2 Bacterial community shifts in healthy versus diseased corals

Chapter 3 describes the introduction a 16S rRNA microarray (PhyloChip) to the field of coral microbiology by characterizing bacterial communities associated with *Montastraea faveolata* (one of the main reef builders in the Caribbean Basin) in healthy corals and corals with symptoms of White Plague Disease type II. The replication of samples from different health states of corals facilitated the testing for statistically significant differences between coral microbiota.

The results of this work were published as a manuscript entitled: "Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata*" in The ISME Journal on January 8, 2009.

A reprint of this publication is included as chapter 3 of this thesis.

1.4.3 Correlations between coral transcriptomes and symbiont genotypes

Chapter 4 describes the first study that has linked differences in the genotypic composition *Symbiodinium* populations to the transcriptomic states of coral hosts. The coral *M. faveolata* is known to associate with multiple *Symbiodinium* strains and was used in a control, heat-stress and stress-recovery experiment. Host and algal symbiont genotyping techniques were combined with measurements of photosynthetic efficiencies and profiling of coral host gene expression using cDNA microarrays. Unexpectedly, the transcriptomic states of corals showed a stronger correlation with the genotypic identity of *Symbiodinium* spp. rather than the experimental conditions tested (i.e., control, stress, and recovery from stress). These results were corroborated by the results of a parallel experiment, performed by Michael DeSalvo, in which the transcriptional states of non-stressed samples from a single coral individual were more similar when they shared the same symbiont genotype.

The results of these two experiments were jointly published as a manuscript entitled: "Coral host transcriptomic states are correlated with *Symbiodinium* genotypes" in the journal *Molecular Ecology* on Feb 8., 2010. A reprint of this publication is included as Chapter 4 of this thesis.

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2 Corals as Threatened Bacterial Habitats

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2.1 Abstract

Contemporary in-depth sequencing of environmental samples has provided novel insights into microbial community structures, revealing that their diversity had been previously underestimated. Communities in marine environments are commonly composed of a few dominant taxa and a high number of taxonomically diverse, low-abundance organisms. However, studying the roles and genomic information of these rare organisms remains challenging, because little is known about their ecological niches and the environmental conditions to which they respond. Given the current threat to coral reef ecosystems, we investigated the potential of corals to provide highly specialized habitats for bacterial taxa including those that are rarely detected or absent in surrounding reef waters. The analysis of more than 350,000 small subunit ribosomal RNA (16S rRNA) sequence tags and almost 2,000 nearly full-length 16S rRNA gene sequences revealed that rare seawater biosphere members are highly abundant or even dominant in diverse Caribbean corals. Closely related corals (in the same genus/family) harbored similar bacterial communities. At higher taxonomic levels, however, the similarities of these communities did not correlate with the phylogenetic relationships among corals, opening novel questions about the evolutionary stability of coral-microbial associations. Large proportions of OTUs (28.7 - 49.1 %) were unique to the coral species of origin. Analysis of the most dominant ribotypes suggests that many uncovered bacterial taxa exist in coral habitats and await future exploration. Our results indicate that coral species, and by extension other animal hosts, act as specialized habitats of otherwise rare microbes in marine ecosystems. Here, deep sequencing provided insights into coral microbiota at an unparalleled resolution and revealed that corals harbor many bacterial taxa previously not known. Given that two of the coral species investigated are listed as threatened under the U.S. Endangered Species Act, our results add an important microbial diversity-based perspective to the significance of conserving coral reefs.

2.2 Introduction

Microorganisms dominate the oceans total biomass (Whitman et al., 1998), phylogenetic diversity and metabolic activity. Only recently have advances in sequencing technology allowed large-scale exploration of taxonomic diversity, population structure, functional potential, and geographic distribution of marine microbes (Pedros-Alio, 2006; Sogin et al., 2006; Yooseph et al., 2007). One of the most remarkable findings has been that diverse microbial taxa exist at very low abundances while accounting for much of the total diversity in various marine environments (Sogin et al., 2006). This rare biosphere stands largely unexplored, but is inherently linked to several important questions (Pedros-Alio, 2007). For example, it is unknown whether low-abundance microbes are restricted to particular environments or universally dispersed (Baas Becking, 1934). In theory, rare organisms may become abundant, if not dominant, in response to environmental or habitat changes, i.e., when conditions shift to become more suitable for rapid growth. However, the exact nature of these changes is yet to be determined. Therefore, the identification of environmental conditions and/or ecological habitats specialized to support rare and underexplored bacteria are critical steps toward a better understanding of the evolutionary mechanisms and ecological forces that drive the biogeography, population structure, and temporal dynamics of the marine microbial biosphere.

Coral reefs are among the most biologically diverse ecosystems in the world, but they are facing an alarming risk of further and more rapid decline (Carpenter et al., 2008). Corals are sessile keystone species in tropical reef environments exposed to tidal mixing and reef water flow (ensuring high dispersal of planktonic microorganisms); thus, they represent a well-suited study system to investigate their role as specialized microbial habitats. Microbial communities in corals have been studied with regard to coral health and disease (Rosenberg et al., 2007; Thurber et al., 2009), antimicrobial properties of coral mucus (Ritchie, 2006; Geffen et al., 2009), and their potential role in recycling organic matter within reef ecosystems (Wild et al., 2004). It has also been hypothesized that microbial partners play important roles in the response (and potentially the adaptation) of the coral holobiont, i.e., the host organism with all its associated microbes, to environmental changes (Reshef et al., 2006).

Diversity surveys using nuclear small subunit ribosomal RNA (16S rRNA) gene clone library sequence data have provided evidence that communities of coral-associated bacteria appear to be host species-specific, and differ from those dominating the surrounding reef water (Frias-Lopez et al., 2002; Rohwer et al., 2002; Bourne and Munn, 2005). One major drawback of these studies is that conventional sequencing methods are limited if the complexity and diversity of microbial community populations are to be captured beyond the most dominant taxa (Dunbar et al., 2002; DeSantis et al., 2007; Sunagawa et al., 2009). With the advent of second-generation sequencing technologies (e.g., pyrosequencing), it is now possible

to detect rare taxa (Sogin et al., 2006) that may serve as a reservoir of functional diversity and potentially becoming dominant when environmental conditions change (Bent and Forney, 2008).

In this study, over 350,000 hypervariable region 6 small subunit ribosomal RNA (V6) tag sequences and 1,960 nearly full-length 16S rRNA gene sequences were generated according to previously published protocols (Huber et al., 2007; Sunagawa et al., 2009). We directly compared bacterial community members living in close association with seven Caribbean coral species and those inhabiting the surrounding seawater to test the potential of corals to provide habitats for marine bacteria. Bacterial communities were also clustered by similarity to determine whether they displayed correlations with the known phylogenetic relationships among the sampled corals. Our study included two Caribbean coral species that are listed as threatened under the U.S. Endangered Species Act to assess the magnitude of biodiversity loss if we fail to preserve imperiled coral species.

2.3 Results and discussion

Corals provide habitats for extremely diverse bacterial communities including rare seawater biosphere members

Ranking of the unique V6-tags sampled from reef water by abundance yielded a low number of dominant taxa, results similar to those previously reported for other marine environments (Sogin et al., 2006; Huber et al., 2007; Pedros-Alio, 2007). More than 50 % of all reads were found in 12 unique V6-tags and a large number of rare taxa accounted for most of the observed diversity (Figure 2.1A, Table S1). A superimposition of tags collected from the seven coral species revealed that many taxa rarely detected in the reef water were highly abundant, and in some cases represented the most dominant taxa in coral samples (Figure 2.1A). Furthermore, we identified more than 20,000 V6-tags that were unique to coral samples (Figure 2.1B). We corroborated the bacterial origin for many of these V6-tags by mapping the tag-sequences to nearly full-length 16S rRNA sequences that were generated from the same set of samples (Figure 2.1A and B). While we cannot discern whether our observations are a consequence of coral-associated bacteria becoming diluted in reef waters or are rare members of the water column finding suitable environments for rapid replication in coral hosts, these results demonstrate that corals provide specialized habitats for select groups of marine bacteria.

Estimates of species richness may be biased by errors in pyrosequencing data (Kunin et al., 2009; Quince et al., 2009) and uneven sampling efforts (Gilbert et al., 2009). Thus, we first re-sampled all sequences to a common depth based on the dataset with the lowest number of reads (15,932 tags, Table S1), and second, clustered V6-tag sequences to the 97 % similarity level ($\text{OTU}_{0.03}$) before comparing the species richness in each coral sample (Table 2.1). Our results suggest that corals potentially harbor several thousand $\text{OTUs}_{0.03}$, which is largely in accordance with

Table 2.1: V6-tag sequencing statistics and species richness estimates

Species / Sample	All tags	All unique tags	Re-sampled*unique tags	OTU _{0.03}	Chao1 _{0.03}
<i>Montastraea faveolata</i>	46,350	5,764	2,681	1,553	2,925
<i>Montastraea franksi</i>	41,962	6,964	3,392	2,050	4,026
<i>Diploria strigosa</i>	40,073	4,908	2,618	1,759	3,801
<i>Acropora palmata</i>	60,390	3,790	2,431	1,671	2,576
<i>Acropora cervicornis</i>	37,095	2,489	2,476	1,616	2,602
<i>Porites astreoides</i>	44,004	4,464	2,048	1,340	3,106
<i>Gorgonia ventalina</i>	36,750	3,322	1,863	1,143	2,177
Reef water	44,190	4,735	2,036	1,079	1,996
total	350,814	27,854	15,023	8,515	14,243

*After removal of chloroplast-derived sequences, V6-reads were re-sampled based on the sample with the smallest number of reads (15,932).

previous diversity estimates (Rohwer et al., 2002; Sunagawa et al., 2009). Corals that form massive, mound-shaped colonies (*Montastraea* spp., *Diploria strigosa*, and *Porites astreoides*) had higher estimated diversities than the branch-forming acroporid species (*Acropora* spp.) and the gorgonian coral *Gorgonia ventalina* (Table 2.1). Therefore, the possibility that morphology plays a role in determining the diversity of coral microbiota becomes an intriguing hypothesis. In total, more than 8,500 OTU_{0.03} were detected and assigned to 31 phyla, with up to 26 phyla per coral species. This census of bacterial taxa in corals with 1,143 - 2,050 OTU_{0.03} detected and 2,177 - 4,026 OTU_{0.03} predicted (Table 2.1) suggests an extraordinary diversity of coral-associated bacteria, which is comparable to the one recently described for sponges (Webster et al., 2009).

Previous studies using clone library sequencing and community profiling data (T-RFLP and DGGE) suggest that bacterial communities in corals are distinct from those inhabiting the surrounding seawater (Frias-Lopez et al., 2002; Bourne and Munn, 2005). With virtually no overlap (e.g., only 5 % of ribotypes were shared between seawater and dead coral surface libraries) between coral-associated and seawater bacteria (Frias-Lopez et al., 2002), and vertical transmission of bacteria seemingly absent in at least one coral species (Apprill et al., 2009), one important question arises: How (and when) do corals acquire the complex microbiota commonly found in adult colonies? Compared to previous studies, our massive sequencing approach has 1) increased the depth of sampling by more than two orders of magnitude, and 2) uncovered a vast number of bacterial taxa associated with an unparalleled number of coral species interrogated for their bacterial communities. More than 4,000 V6-tags were shared in coral and seawater libraries (Figure 2.1A), thus a lack of sequencing depth was a likely reason for the apparent absence of overlap between coral- and seawater-associated bacteria in previous studies. The detection of rare taxa in the water column, while being dominant in

corals, has also important implications for the mode of bacterial symbiont acquisition in coral hosts. Given our results, it is reasonable to hypothesize that rare seawater biosphere members act as seed organisms for coral microbiota, implying that corals could acquire species-specific bacteria from the environment without any vertical mode of transmission. The possibility to capture the presence of rare taxa will thus be extremely valuable in studying both the onset of coral-microbial associations and the spatio-temporal variability in coral-associated microbiota.

Similarities of bacterial communities based on V6-tag abundance profiles

Reef-building corals (Hexacorallia:Scleractinia) are divided into two main phylogenetic lineages (i.e., Short/Robust and Long/Complex clades), which are separated by approximately 240-288 million years of divergent evolution (Romano and Palumbi, 1996; Medina et al., 2006). We clustered V6-tag abundance profiles to ask whether a correlation exists between the bacterial community composition and the known coral host phylogeny (Figure 2.1C). Similarities between bacterial communities were found to partially reflect the known coral phylogeny, but also showed inconsistencies at higher taxonomic levels. For example, the most similar bacterial communities among all samples were derived from the congeneric species *Acropora palmata* and *A. cervicornis*. Furthermore, samples clustered by their clade affiliation, i.e., *Montastraea faveolata*, *M. franksi*, and *Diploria strigosa* (Short/Robust clade) were found in a different cluster than the more distantly related acroporid relatives (Long/Complex clade). Conversely, we found the octocoral-derived bacterial community to be inconsistent (i.e., clustering with Short/Robust clade communities) with the currently accepted phylogenetic placement of gorgonians in the anthozoan tree (i.e., outside Scleractinia). Likewise, *Porites astreoides* did not cluster with the acroporid species, although they belong to the same clade (Long/Complex).

Understanding of the acquisition, maintenance, and change of microbial communities in a coral host are fundamental questions in the study of coral-microbial associations. Vertical transmission of bacteria has, at least for one coral species, been excluded as a mode of symbiont acquisition (Apprill et al., 2009), implying that corals may need to acquire their complex microbiota solely by environmental (horizontal) transmission throughout their lifespan. Thus, adult corals are likely to be associated with a mixed consortium of bacteria of which many may not co-evolve with their host. In fact, Littman et al. (2009) compared bacterial communities of three Indo-Pacific acroporid species at two environmentally distinct locations using DGGE, T-RFLP, and 16S RNA gene clone library sequencing, and found that samples grouped by location rather than coral species (Littman et al., 2009). Thus, environmental factors appear to play an important role in driving coral-bacterial community composition, which is in accordance with temporal variations that had previously been reported for a Mediterranean coral species (Koren and Rosenberg, 2006). Our data did not allow for assessing inter-colonial variability, i.e., whether replicate samples from different corals would have clustered

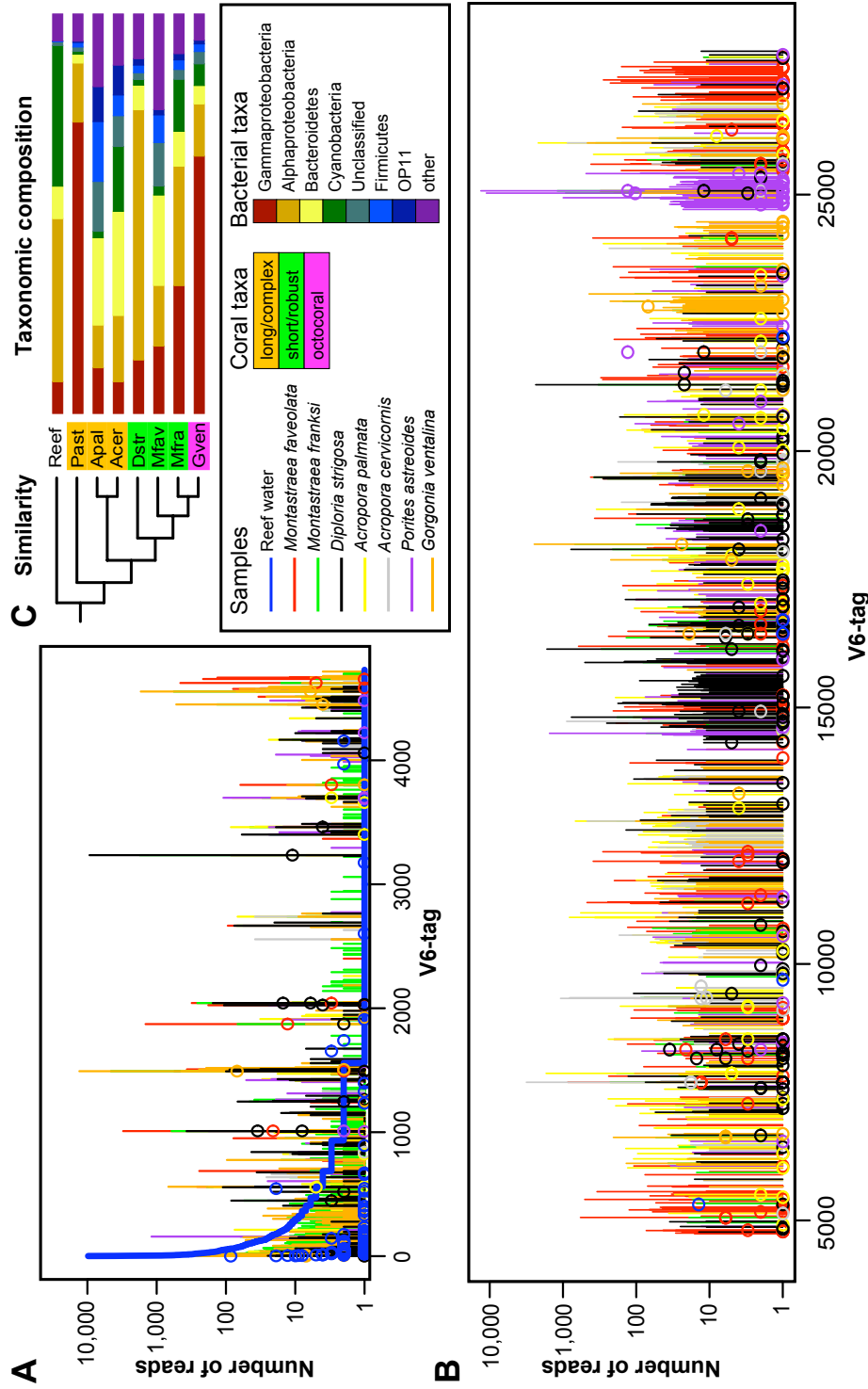


Figure 2.1: V6-tag abundance profiles, similarity clustering, and taxonomic composition of bacterial communities. (A) Rank abundance curve for V6-tags detected in reef water superimposed with abundances found in coral samples shown as vertical colored bars. (B) Abundances of V6-tags, which were detected exclusively in corals, are shown alphabetically sorted by taxonomic classification of V6-tags (x-axis). In (A) and (B), circles denote log-scaled abundances of nearly full-length 16S rRNA sequences that were mapped to the respective V6-tag sequences. (C) Taxonomic composition of all samples and dendrogram of OTU abundances showing similarities between samples, which are color-coded according coral host taxonomy. Details on the taxonomic composition of each sample can be found in Table S1. Abbreviations used: Acer=*Acropora cervicornis*; Apal=*Acropora palmata*; Dstr=*Diploria strigosa*; Gven=*Gorgonia ventalina*; Mfav=*Montastraea faveolata*; Mfra=*Montastraea franksi*; Past=*Porites astreoides*; Reef=reef water.

according to species. However, it is intriguing to note that an averaged, abundance-based index of community composition displayed similar profiles among closely related corals in the same genus (*Acropora* spp.) or family (*Montastraea* spp. and *D. strigosa*), but not at higher taxonomic levels. Thus, our results lend further support to the idea that coral-associated microbiota are similar among closely related coral species. Interestingly, coral-associated microbial communities were also found to show higher similarities in metabolic characteristics within groups of congeneric coral species (reviewed in Ritchie and Smith, 2004), while the topology of the clusters was found to be inconsistent with the higher clade designations of reef-building corals (e.g., the Short/Robust clade corals in the genus *Diploria* were grouped together with clusters of species belonging to the Long/Complex clade genera *Acropora* and *Porites*). Based on this information, corals may represent specialized habitats in which microorganisms have divergently evolved over many generations (e.g., due to the availability of nutrients specific to each coral host), but coral-microbial associations may not be stable over long evolutionary time scales. More focused work is required in order to 1) better understand the ecological and evolutionary forces that drive coral-associated bacterial community composition, and 2) identify specific bacterial species that may be co-evolving with their hosts as has been exemplified in a number of animals including cnidarians (Taylor et al., 2004; Moran, 2006; Dethlefsen et al., 2007; Fraune and Bosch, 2007; Ley et al., 2008a).

Threatened coral species host unique and underexplored bacterial taxa

We examined whether different coral species provide distinct microbial habitats by testing the null-hypothesis that OTUs were randomly present or absent in any of the 8 samples. We binned unique OTUs_{0.03} into the 28 (=256) possible permutations (e.g., V6-tags present in reef water only, tags present in reef water and *M. faveolata*, etc.) and found that among all possible combinations, those that represent the presence of OTUs in one particular environment only (e.g., present in reef water only, in *M. faveolata* only, etc.) ranked at the top 8 positions (Table 2.2). This result significantly deviates from a random distribution, and supports the hypothesis that coral species are likely to harbor a number of host-specific bacteria (Rohwer et al., 2002). A search for the most closely related relatives to the most abundant coral-associated bacteria revealed that corals harbor many unclassified species and potentially novel genera or even higher taxonomic groups (Table 2.3). In the future, a sampling strategy that includes multiple individuals of the same coral species in different environments will help 1) delineating host species-specific taxa from individual-to-individual variability, and 2) determining the role of environmental factors on the diversity and community structure of coral microbiota.

Host-associated bacterial communities can be shaped by a number of biological and physicochemical factors. For example, in humans, inter-microbial competition, pH and the activity of the immune system are all known to influence

Table 2.2: Top 10 ranked OTU_{0.03} distribution possibilities

OTU _{0.03} present in	Number of OTU _{0.03}	Number (%) of all OTU _{0.03} in sample/s
<i>Diploria strigosa</i> only	907	1,902 (47.7)
<i>Acorpora palmata</i> only	866	1,834 (47.2)
<i>Acropora cervicornis</i> only	768	1,793 (42.8)
<i>Montastraea faveolata</i> only	757	1,754 (43.2)
<i>Porites astreoides</i> only	701	1,429 (49.1)
<i>Montastraea franksi</i> only	636	2,448 (26.0)
Reef water only	601	1,183 (50.8)
<i>Gorgonia ventalina</i> only	383	1,333 (28.7)
<i>Montastraea franksi</i> and Reef water	202	3,193 (6.3)
<i>Montastraea franksi</i> and <i>Diploria strigosa</i>	180	3,772 (4.8)

Table 2.3: Best non-coral associated BLAST hits of most abundant OTUs isolated from corals

OTU _{0.03} *	Accession	Best BLAST hit	identity (%)	Isolation source	Accession
Mfav_H04	GU118607	Endosymbiont of <i>Acanthamoeba</i> sp.	93	Protozoa	AF215634
Mfav_L18	GU118673	<i>Flexibacter aggregans</i>	94.2	Sand	AB078038
Mfra_I15	GU118732	Endosymbiont of <i>Acanthamoeba</i> sp.	93	Protozoa	AF215634
Mfra_G19	GU118699	Uncultured bacterium clone AG3	96.7	Fish	EU884929
Apal_J06	GU118139	<i>Desulforhopalus singaporensis</i>	97.3	Marine mud	AF118453
Apal_K21	GU118042	Uncultured bacterium clone 655952	87.6	Sediment	DQ404824
Acer_F19	GU117998	<i>Leptolyngbya</i> sp. 0BB32S02	94.6	Freshwater	AJ639894
Acer_F06	GU117952	<i>Flexibacter aggregans</i>	94.3	Sand	AB078038
Past_G20	GU119165	Uncultured bacterium clone AG3	96.7	Fish	EU884929
Past_A20	GU118916	Uncultured <i>Desulfocapsa</i> sp. clone CBII115	95.5	Marine sediment	DQ831556
Gven_C04	GU118411	<i>Spongiobacter nickelotolerans</i>	93.6	Marine sponge	AB205011
Gven_C22	GU118496	<i>Aquaspirillum peregrinum</i> subsp. integrum	86.5	Shellfish	EF612768
Dstr_B21	GU118204	Uncultured bacterium clone P9X2b3F06	94.2	Seafloor lava	EU491139
Dstr_G05	GU118184	Uncultured alpha proteobacterium HOC19	99.5	Marine sponge	AB054153

*selected cluster representative

the formation of the microbiota (Kuramitsu et al., 2007; Ley et al., 2008b). Likewise in corals, antimicrobial activity has been detected and suggested to play a role in the regulation of microbial populations (Ritchie, 2006; Geffen et al., 2009). In addition, it is important to notice that reef-building corals live in symbiosis with photosynthetic dinoflagellates, which are responsible for strong diurnal fluctuations in pH and O₂ concentrations within coral tissues (Kühl et al., 1995; Shashar et al., 1993). These parameters change along a steep gradient ranging from the coral tissue layer across a diffusive boundary layer that separates the coral tissue microenvironment from the surrounding seawater (Kühl et al., 1995; Shashar et al., 1993). We suggest that a combination of coral host immunity, inter-microbial interactions, physicochemical parameters (e.g., nutrients, pH, O₂), and other factors, are likely to create adaptive landscapes that explain the ecological niche partitioning observed in this study. Furthermore, the adaptation to physico-chemically distinct microenvironments in different coral hosts over evolutionary time scales has potentially generated an untapped source of genomic innovation.

Conclusions

At the beginning of the 21st century, we find ourselves in an era where second-generation sequencing technologies have exposed a vast amount of undiscovered microbial diversity governing the Earth's biosphere. The high rate of discoveries ironically parallels the worldwide decline of many ecosystems, including coral reefs. Often considered as the proverbial canary in the mine for the oceans, their survival is currently at stake (Carpenter et al., 2008), while the ecological role and genomic makeup of the coral microbiome still remain largely unknown. Here, the application of 16S pyrotag sequencing revealed an extraordinary bacterial diversity in reef-building coral species, and provided first insights into the link between coral host communities and rare seawater biosphere members. Inconsistencies in the correlation between bacterial community composition and coral host phylogenies emphasized the need to better understand the ecological and evolutionary forces that determine the structure and composition of coral microbiota. The application of massively-parallel tag sequencing, along with hybridization-based methods (Sunagawa et al., 2009) and metagenomics studies (Wegley et al., 2007; Vega Thurber et al., 2008; Thurber et al., 2009), represents a promising tool to complement established methods in rapidly advancing our knowledge about the ecological role, population structure, and dynamics of microbial communities in healthy vs. stressed/diseased states of corals (Bourne et al., 2009).

Our study included two coral species, *Acropora palmata* and *A. cervicornis*, that once represented the most abundant species in Caribbean coral reefs, but are now listed as threatened under the U. S. Endangered Species Act. Given that our results are based on only a handful of coral species, it is reasonable to predict that future sequencing of coral- and other host-associated microbiota is likely to uncover a wealth of yet unknown genetic and functional diversity. Thus, our findings substantiate the justification (Costanza et al., 1997) and highlight

the importance of conserving coral reefs as an underexplored ecosystem from a microbial diversity-based perspective.

2.4 Materials and methods

Ethics statement

Experimental research followed internationally recognized guidelines according to CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) permit numbers: SEX/A-26-08 and SE/A-13-08. No ethical approval was required for any of the experimental research described here.

Sample collection and isolation of nucleic acids

On the 26th and 27th of March 2008, replicate samples (N=5) from seven Caribbean coral species (depth range: 1.5 - 5.5 m) were collected together with a reef water sample at "Crawl Cay" reef near Bocas del Toro in Panamá (9°15'N; 82°07'W) by SCUBA using a hammer and chisel. All collected coral fragments (size: 1 - 4 cm²) were placed in plastic bags, rinsed with 0.22 μm filtered seawater, and flash frozen in a dry shipper within less than one hour after collection. The reference water sample was collected (depth: \sim 1 m) at the Crawl Cay buoy using a 5 L plastic bottle and filtered using a 0.22 μm SterivexTM filter unit (Millipore), which was immediately flash frozen after filtration. At UC Merced, the coral fragments were homogenized on dry ice using a mortar and pestle and the frozen filter cut into smaller pieces (\sim 25 mm²). Approximately 50 mg of the resulting coral powder or the frozen filter pieces were used to extract DNA using the PowerPlant DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA). To increase the yield and quality of DNA preparations, we modified the manufacturers instructions by: 1) adding 0.19 μL lysozyme (Epicentre; final: 10 U μL^{-1}) to the Bead Solution/sample mixture, followed by an incubation of 10 min at room temperature, 2) adding 25 μL proteinase K (Invitrogen; final: \sim 0.8 mg mL⁻¹) to the lysozyme-treated mixture, followed by an incubation for 60 min at 65 °C, and 3) adding 400 mg of each 0.1 and 0.5 mm zirconia/silica beads before samples were homogenized for 30 s using a Mini-BeadBeater-8 (Biospec Products, Inc., Bartlesville, OK, USA) instead of a Vortex Adapter (MoBio).

V6-tag and nearly full-length sequence generation

The amplification of V6-tag amplicons from total DNA of coral samples required optimizations of previously published methods (Sogin et al., 2006; Huber et al., 2007). After pooling replicate DNA extractions of each sample/species, we used Platinum High-Fidelity Taq Polymerase (Invitrogen) in combination with Buffer G (Epicentre), 50 ng of template DNA and keyed primers (Huber et al., 2007) in triplicate 30 μL PCR reactions and ran the following cycling conditions: [94 °C - 2', (94 °C - 30", 56 °C - 30", 68 °C - 30") x 30, 68 °C - 2']. The tripli-

cate reactions were pooled and amplicons purified (Qiagen), before they were run out on a 2 % agarose gel, excised (Qiagen), and purified to remove non-specific amplification products. Samples were sent to the Marine Biological Laboratory in Woods Hole, MA, for pyrosequencing. The resulting data were processed in February 2009 according to previously published methods (Sogin et al., 2006; Huber et al., 2007). V6-tag sequences have been deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) under the project number SRP001172 and are publicly available from the Visualization and Analysis of Microbial Population Structure (VAMPS) database at <http://vamps.mbl.edu>.

In addition to V6-tag amplicons, nearly full-length 16S rRNA gene amplicons were generated from each sample and clone libraries generated following standard methods (Sunagawa et al., 2009). Transformed clone library stocks were sequenced by Agencourt Bioscience.

Sequence data processing, clustering, and diversity estimates

Chromatograms of nearly full-length 16S rRNA gene sequences were assembled, trimmed, quality-checked, and chimeric sequences removed as previously described (Sunagawa et al., 2009). The resulting sequences were classified using two different pipelines (STAP (Wu et al., 2008) and RDP (Wang et al., 2007)), which were identical in their outcome regarding the identification of chloroplast-derived sequences (data not shown). All nearly full-length sequences were deposited to the DDBJ/EMBL/GenBank databases under accession numbers: GU117926-GU119887.

Unique V6-tag sequences and their abundance data for each sample were downloaded from the VAMPS database. A local MySQL database was updated, and queried at different stages of the analysis to obtain general statistics or data matrices for further analyses. After downloading the processed sequence data, misclassified (as Firmicutes) chloroplast-derived V6-tags were identified by mapping all tags to nearly full-length sequences and subsequently removed. The rank abundance curve for tags detected in seawater was plotted and superimposed with tags present in coral samples using functions in R. V6-tags that were detected in clone library-derived sequences were identified as described above and their quantities visualized in Figure 2.1 A and B.

In order to account for uneven sampling efforts, V6-tags were re-sampled to a common total of 15,932, i.e., the number of unique sequences in the sample with the lowest number of sampled tags, using *Daisy_chopper* (v1.0) (Gilbert et al., 2009). Re-sampled sequences were aligned and a pair-wise distance matrix calculated with the software programs MUSCLE (Edgar, 2004) and *quickdist* (Howe et al., 2002; Sogin et al., 2006). V6-tags were clustered into operational taxonomic units at the 97 % similarity level ($OTU_{0.03}$) and Chao1 nonparametric richness estimates calculated using the program DOTUR (Schloss and Handelsman, 2005).

Sample clustering and distribution of $OTUs_{0.03}$

Determining the similarities of bacterial communities based on V6-tags us-

ing phylogenetic methods (Hamady et al., 2009) was not feasible since many tags had no significant match to any sequence in the complete Greengenes (DeSantis et al., 2006b) (45 %; release November 2008) or Silva (Pruesse et al., 2007) databases (31 %; release 100). Thus, we clustered V6-tags based on OTU_{0.03} data using Bray-Curtis distances and inferred a dendrogram with the unweighted pair-group average algorithm (UPGMA) as implemented in the NEIGHBOR program of the PHYLIP package (Felsenstein, 1989). The taxonomic composition of samples are based on the V6-tag classifications provided by the VAMPS database and were visualized using iTOL (Letunic and Bork, 2007). For the distribution of OTUs_{0.03} among all samples, we assigned one of 256 (=28) possible combinations of presence or absence to each OTU_{0.03}, and ranked the combinations by OTU abundance.

Similarity searches of most abundant 16S rRNA sequences

For similarity searches of 16S rRNA gene sequences, all sequences within each sample were aligned using NAST (DeSantis et al., 2006a), a distance matrix calculated using the DNADIST tool of the PHYLIP package through the Greengenes website (DeSantis et al., 2006b), and clustered to the 97 % similarity level as described above. The best cluster representative was identified based on sequence length and average quality values. A BLASTn (megablast) search was performed against the GenBank nucleotide database to identify the most similar non-coral-derived sequence entry.

Bioinformatics

Data were analyzed using MySQL database queries, custom and published (Gilbert et al., 2009) Perl scripts, and UNIX commands (custom commands and scripts available upon request). Statistical analyses were performed in the R software environment (Ihaka and Gentleman, 1996) and visualization of data was supported by R scripts and iTOL (Letunic and Bork, 2007).

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2.7 Supporting information

Supporting information is available online at:
<http://dx.plos.org/10.1371/journal.pone.0009554>

2.8 Chapter acknowledgement

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3 Bacterial Community shifts in Diseased *Montastraea faveolata*

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3.1 Abstract

Increasing evidence confirms the crucial role bacteria and archaea play within the coral holobiont, that is, the coral host and its associated microbial community. The bacterial component constitutes a community of high diversity, which appears to change in structure in response to disease events. In this study, we highlight the limitation of 16S rRNA gene (16S rDNA) clone library sequencing as the sole method to comprehensively describe coral-associated communities. This limitation was addressed by combining a high-density 16S rRNA gene microarray with, clone library sequencing as a novel approach to study bacterial communities in healthy versus diseased corals. We determined an increase in diversity as well as a significant shift in community structure in *Montastraea faveolata* colonies displaying phenotypic signs of White Plague Disease type II (WPD-II). An accumulation of species that belong to families that include known coral pathogens (Alteromonadaceae, Vibrionaceae), bacteria previously isolated from diseased, stressed or injured marine invertebrates (for example, Rhodobacteraceae), and other species (for example, Campylobacteraceae) was observed. Some of these species were also present in healthy tissue samples, but the putative primary pathogen, *Aurantimonas coralllicida*, was not detected in any sample by either method. Although an ecological succession of bacteria during disease progression after causation by a primary agent represents a possible explanation for our observations, we also discuss the possibility that a disease of yet to be determined etiology may have affected *M. faveolata* colonies and resulted in (or be a result of) an increase in opportunistic pathogens.

3.2 Introduction

Reef-building corals are associated with a dynamic, highly diverse consortium of microorganisms that includes protists, bacteria, archaea and endolithic algae and fungi (Shashar and Stambler, 1992; Bentis et al., 2000; Rohwer et al., 2002; Baker, 2003; Kellogg, 2004; Wegley et al., 2004; Rosenberg et al., 2007; Harel et al., 2008). To study the phylogenetic diversity of the bacterial and archaeal components, sequencing the 16S rRNA gene (16S rDNA) is commonly used because of its ability to identify the species without the need for laboratory cultivation. Representative studies have uncovered that (i) different corals appear to harbor distinct and highly diverse bacterial communities (Rohwer et al., 2002; Bourne and Munn, 2005), (ii) the overlap between bacteria inhabiting the water column and the coral host is small (Frias-Lopez et al., 2002) and (iii) the community of coral-associated bacteria undergoes changes in response to stress or disease (Cooney et al., 2002; Frias-Lopez et al., 2002; Pantos et al., 2003; Pantos and Bythell, 2006). Despite these advances, only a handful of primary coral pathogens have been identified to date (reviewed in Rosenberg et al., 2007). Furthermore, the description of many coral diseases is often confounded by the lack of clear diagnostic criteria so that similar disease signs may emerge in multiple coral species, whereas a putative pathogen has only been verified for one or a subset of species (Richardson, 1998; Pantos et al., 2003; Sutherland et al., 2004). For example, White Plague Disease type II (WPD-II) has been reported to affect more than 40 different coral species (Weil et al., 2006), whereas the bacterial pathogen *Aurantimonas coralllicida* isolated from *Dichocoenia stokesi* (Richardson et al., 1998a,b; Denner et al., 2003) is the only example for which Kochs postulates have been fulfilled.

It is known that diseases may result from complex interactions between host, causative agent(s) and the environment (Martin et al., 1987) and it has been suggested that profiling the host microbiota will play an important role in better understanding coral diseases (Work et al., 2008). The technology of 16S rRNA gene microarrays performs massively parallel assays in a single experiment (Gentry et al., 2006), and thus represents a powerful tool to profile host microbiota at different health states. The most comprehensive 16S rRNA gene microarray to date (PhyloChip G2) consists of approximately 300 000 oligonucleotide probes assaying 8741 operational taxonomic units (Wilson et al., 2002; DeSantis et al., 2007). Although this technology may not be suitable for discovering novel taxa, recent studies have shown both its advantage over 16S rDNA library sequencing (DeSantis et al., 2007) and applicability to a variety of environmental samples including subsurface water, urban aerosols or uranium contaminated soil (Brodie et al., 2006, 2007; DeSantis et al., 2007). Introducing this cost-effective technology to the field of coral microbiology may not only help to better understand the diversity and community structure of coral-associated microbiota, but also to delineate various pathologies with similar disease signs but different etiologies. In this study, PhyloChip hybridization data combined with 16S rDNA clone library sequences

from a single colony of the Caribbean coral *Montastraea faveolata* suggests that corals represent an under-sampled environment, which can be expected to include a high level of novel bacterial species. A combined analysis of PhyloChip and 16S rDNA sequence data using *M. faveolata* samples that displayed phenotypic signs of WPD-II following a coral bleaching episode in 2005 revealed a significant shift in community structure in response to disease, with an accumulation of ribotypes that were similar to pathogens or bacteria previously isolated from diseased, injured or stressed marine invertebrates. The putative primary pathogen *A. corallicida*, however, was not identified. Based on our results, we discuss the possibility that *M. faveolata* colonies may have been affected by a disease of yet unknown etiology that may have resulted in (or be a result of) an increase in opportunistic pathogens.

3.3 Materials and methods

Sample collection

For in-depth sequencing of bacteria associated with *Montastraea faveolata*, we selected one coral fragment (collected near Isla San Cristobal in August 2006 at Bocas del Toro, Panamá) that was acclimated in a seawater tank for 23 days and shipped frozen to the University of California Merced (Merced, USA). *M. faveolata* colonies displaying visible signs of White Plague Disease type II (Richardson et al., 1998a), were sampled at two reefs, 'Turrumote' (17°56.097' N, 67°01.130' W) and 'The Buoy' (17°56.038' N, 66°59.090' W), off La Parguera on the southwest coast of Puerto Rico during disease outbreaks after the 2005 bleaching event in January (Turrumote: WP1-J, WP2-J) and May 2006 (The Buoy: WP3-M, WP4-M), respectively. In parallel, control samples from healthy looking colonies were collected (H1-J, H2-M, H3-M, H4-M). All samples were collected using a hammer and chisel at 10-20 m depth and immediately frozen in liquid nitrogen. Care was taken to keep seawater inclusion to a minimum.

DNA extraction and PCR amplification of 16S rDNA

On dry ice, coral tissue/skeleton was chiseled off the first 0.5 - 1 cm from the surface and ground to powder using a sterile mortar and pestle. The lesion boundary in diseased corals was sampled so that approximately equal amounts originated from healthy looking and white, tissue-devoid parts. For in-depth sequencing, about 125 mg of powder was added to 600 μ l cell lysis buffer (100 mM NaCl, 100 mM Tris-Cl, 25 mM EDTA, 0.5 % SDS, 500 μ g ml⁻¹ Proteinase K, pH 8.0) before incubation at 55 °C for 16 h. 8 μ l RNase A (1 mg ml⁻¹) was added before incubation at 37 °C for 60 min. Proteins and skeletal debris were removed after adding 1/3 vol. protein precipitation solution (4 M guanidine thiocyanate, 0.1 M Tris-Cl, pH 7.5) and centrifugation at 16,000 g for 5 min. The DNA was recovered after precipitation using 1 vol. isopropanol and two ethanol

(70 %) washes. PCR conditions were the same as described below with the exception of using the reverse primer 1492R(Y) (5'-CGGYTACCTTGTTACGACTT). For healthy and diseased tissue samples, approximately 50 mg of powder was applied to the PowerPlant DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA), which removed PCR inhibitors more efficiently than other protocols (phenol/chloroform or proteinase K/guanidine thiocyanate-based protocols, UltraSoil kit (MoBio), CTAB/PVP-based protocols). We modified the manufacturers instructions by: (a) adding lysozyme (Epicentre; final: 10 U μl^{-1}) to the Bead Solution/sample mixture, followed by an incubation of 10 min, (b) adding 25 ml proteinase K (20 mg ml^{-1}) to the lysozyme-treated mixture, followed by incubation at 65 °C for 10 min and (c) bead-beating on a Vortex Adapter (MoBio) for 15 instead of 10 min.

For the amplification of 16S rRNA genes, we used 50 ng DNA and universal bacteria-specific primers 27F (5'-AGAGTTTGTATCCTGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT) in 25 μl PCRs containing 12.5 μl Buffer G (Epicentre), 1 μM of each primer and 2.5 U REDgDNA Taq polymerase (Sigma-Aldrich, St Louis, MO, USA). We ran and pooled gradient PCRs [95 °C - 3min; 95 °C - 1 min; 48-62 °C - 1 min, 72 °C - 2 min (25 x); 72 °C - 10 min] to increase the diversity of amplified 16S rRNA genes.

16S rDNA cloning, sequencing, assembly, classification and annotation

One 16S rDNA library (N=943) for in-depth sequencing of *M. faveolata*-associated bacteria and pooled libraries from four healthy (H) (N=317) and four diseased (N=340) samples were generated using the TOPO-TA cloning kit (pCR4-TOPO vector, Invitrogen). Selected clones were bi-directionally sequenced using the primers T3 (5'-ATTAACCCTCACTAAAGGGA) and T7 (5'-TAATACGACTCACTATAGGG) on an ABI3700 sequencer (Applied Biosystems, Foster City, CA, USA) at the DOE Joint Genome Institute (<http://www.jgi.doe.gov/>). Bases were called using Phred (Ewing et al., 1998; Ewing and Green, 1998) and processed by Perl scripts that sequentially assembled the reads into 16S rDNA contigs, removed vector sequence, end-trimmed low quality bases and removed short as well as overall low quality sequences.

For the comparison of clone library and Phylo- Chip data, sequences were processed through tools available at <http://greengenes.lbl.gov> (DeSantis et al., 2006b). Briefly, 16S rDNA sequences were aligned using NAST (DeSantis et al., 2006a) and chimera-checked using Bellerophon (Huber et al., 2004). Similarities to publicly available sequences were calculated using the DNADIST tool of the Phylip package [DNAML-F84 option; transition/ transversion ratio: 2.0; A, C, C, G frequencies: 0.2537, 0.2317, 0.3167 and 0.1979, respectively; lane mask (Lane, 1991) used]. For classification purposes, sequences were assigned to the taxonomic ranks 'Phylum', 'Class', 'Order', 'Family', 'Subfamily' and OTU (operational taxonomic unit), when the similarity to database records was equal to or higher than 80, 85, 90, 92, 94 and 97 %, respectively (DeSantis et al., 2007).

For class level comparison of clone library sequences from healthy and diseased tissues, sequences were classified as described above as well as according to nearly full-length 16S rDNA sequences using tools of the Ribosomal Database Project II (Cole et al., 2007). Sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers: FJ202063-FJ203662.

Species richness estimates

A clone distance matrix was generated online at <http://greengenes.lbl.gov> and used to calculate nonparametric richness estimations Chao1 (Chao, 1984) and ACE (Chao and Lee, 1992) using the software DOTUR (Schloss and Handelsman, 2005) and furthest-neighbor as the clustering algorithm.

PhyloChip hybridizations

PCR amplicons from each of the four healthy and four diseased coral samples were hybridized on PhyloChips (G2). DNA quantification, fragmentation, addition of internal standards, labeling, PhyloChip hybridization, staining and scanning were performed as previously described (Brodie et al., 2006). Detailed information on oligonucleotide probe selection and array design can be found in Brodie et al. (2006, 2007). Probe pairs scored as positive were those that met two criteria: (i) the intensity of fluorescence from the perfectly matching probe was greater than 1.3 times the intensity from the mismatching control and (ii) the difference in intensity, perfectly matching minus mismatching, was at least 130 times greater than the squared noise value ($> 130N^2$). When summarizing PhyloChip results, all positive probe sets ($pF \geq 0.9$) at the OTU level were summarized to the bacterial family level (92 % similarity).

Data analysis

Normalized intensity values were \log_2 transformed before PhyloChip hybridization data were analyzed using the TM4 software (Saeed et al., 2003). Hierarchical clustering was done using the average linkage method and Euclidean distance matrix. The Kolmogorov-Smirnov test for equality of variances between healthy and diseased samples was performed in SigmaStat (Systat Software Inc.) and failed ($P < 0.05$). Consequently, differentially abundant OTUs between healthy and diseased samples were tested using an unpaired t-test assuming unequal variance (Welch's approximation).

3.4 Results

*Diversity and novelty of bacteria associated with *Montastraea faveolata**

The sequenced clone library ($N = 943$) from a single *Montastraea faveolata* colony represented more than 10 times the data reported for libraries in previous coral microbiological studies (Frias-Lopez et al., 2002; Rohwer et al., 2002; Bourne

and Munn, 2005; Pantos and Bythell, 2006; Barneah et al., 2007). Here, 178 unique OTUs were detected and diversity estimates ranged from 307 to 329 ribotypes according to Chao1 and ACE, respectively. The rarefaction curve did not reach an asymptote (Figure 3.1) indicating insufficient sampling to capture the total diversity of the bacterial community.

Based on nearly full-length sequences, only 7.0 % of the 16S rDNA sequences were classified at the OTU level (Table 3.1). At a decreasing taxonomic resolution, however, the number of classified sequences increased continuously to 99.7 % at the phylum level. For example, more than 70 % of the 16S rDNA sequences could be classified at the family level. When compared with clone library sequencing, PhyloChip hybridizations detected a higher richness at all levels of taxonomic resolution. The taxonomic categories detected by cloning were generally found as a subset of those reported from the hybridization experiment (Table 3.1). As an exception to this trend, none of the 21 OTUs detected by clone library sequencing were reflected by the corresponding PhyloChip data indicating a high degree of yet uncharacterized species in coralassociated bacteria.

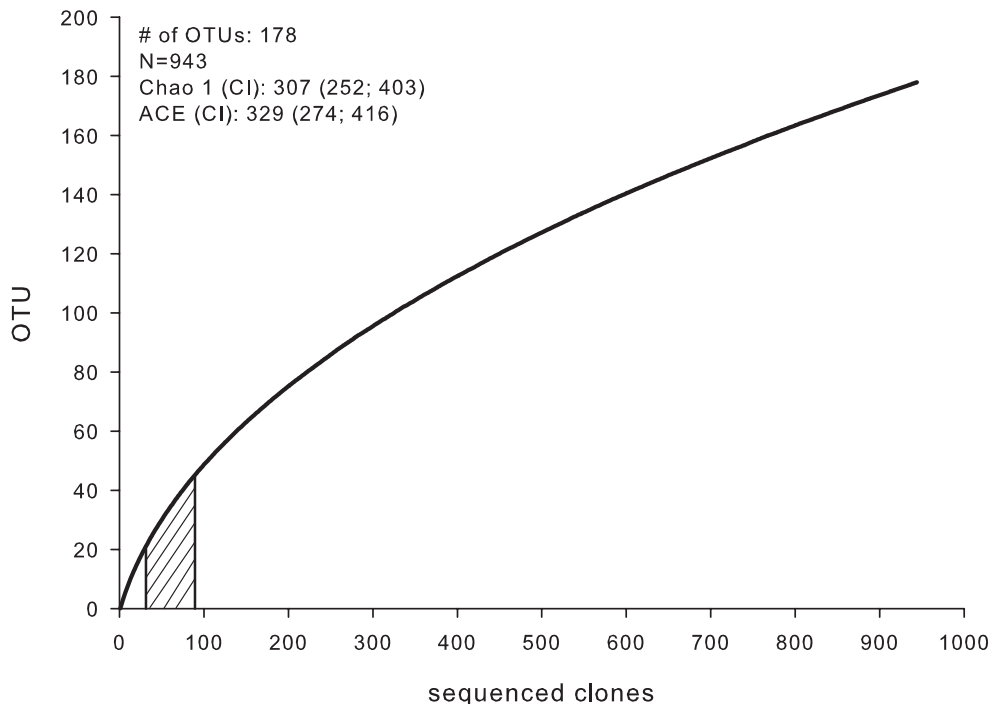


Figure 3.1: Rarefaction analysis for a recombinant 16SrDNA clone library ($n = 943$) generated from a single *Montastraea faveolata* colony. Patterned area shows the range of typical clone library sizes from selected coral microbiological studies [(Frias-Lopez et al., 2002; Rohwer et al., 2002; Bourne and Munn, 2005; Pantos and Bythell, 2006; Barneah et al., 2007)]. Estimation of species diversity (Chao1) and abundance-based coverage estimation (ACE) are shown with 95 % confidence intervals (CI). Distance matrix was generated online at <http://greengenes.lbl.gov>; cluster distance 0.03; rarefaction curve generated using the software DOTUR (Schloss and Handelsman, 2005).

Comparison of bacterial diversity in healthy and diseased coral samples

PhyloChip data from healthy ($N = 4$) and diseased ($N = 4$) coral samples were pooled according to health state and analyzed for abundances of unique taxa in only healthy, or only diseased samples (Table 3.2). At all taxonomic levels, the majority of members could be found in both healthy and diseased coral samples. The relative abundance of taxon members in diseased corals was always higher than in healthy corals. For example, out of 8741 OTUs assayed by the current generation of the PhyloChip (G2), a total of 1702 were detected ($pf \geq 0.9$) across all arrays ($N = 2 \times 4$). Approximately two-thirds of these OTUs were detected in both healthy and diseased states, 30.8 % were unique to diseased corals and only 2.5 % unique to healthy corals (Table 3.2). This trend suggests a higher level of diversity in bacterial community composition associated with the diseased samples.

Table 3.1: Number of distinct taxonomic ranks identified by PhyloChip and/or clone library sequencing in a single *M. faveolata* sample

Taxonomic rank (% cutoff)	Clones classified/not detected by PhyloChip (%)	Count of taxa detected by:		
		Chip only (%)	Chip and clone (%)	Clone only (%)
Phylum (≥ 80)	99.7 ^a /0.5	27 (67.5)	13 (32.5)	0 (0)
Class (≥ 85)	95.5/4.7	57 (72.1)	22 (27.9)	0 (0)
Order (≥ 90)	79.5/20.7	113 (74.3)	37 (24.3)	2 (1.3)
Family (≥ 92)	70.4/29.7	221 (83.4)	43 (16.2)	1 (0.4)
Subfamily (≥ 94)	48.9/51.2	295 (90.8)	29 (8.9)	1 (0.3)
OTU (≥ 97)	7.0/93.0	1441 (98.6)	0 (0)	21 (1.4)

The DNAML-F84 homology cutoff used for taxonomic rank assignment of 16S rDNA clones is shown in parentheses. Percentages of classifiable and not detected 16S rDNA clones are based on the total number of sequenced clones ($N = 943$). Counts and percentages of distinct taxonomic rank members detected by PhyloChip hybridization, clone library sequencing and a combination of both methods are shown.

^aThree sequenced clones (0.3 %) could not be classified at a level of ≥ 80 % to the reference set. (FJ202339, FJ202502, FJ202893).

Table 3.2: Number of taxonomic rank members detected in replicate hybridizations from healthy and diseased coral samples

Taxonomic rank (% cutoff)	Healthy (abs.)	Diseased (abs.)	Increase (%)	Healthy only (%)	Diseased only (%)	Healthy and diseased (%)	Total
OTUs (97)	1178	1659	40.8	43 (2.5)	524 (30.8)	1135 (66.7)	1702
Subfamilies (94)	306	342	11.8	7 (2.0)	43 (12.3)	299 (85.7)	349
Families (92)	244	269	10.2	6 (2.2)	31 (11.3)	238 (86.5)	275
Orders (90)	146	153	4.8	5 (3.2)	12 (7.6)	141 (89.2)	158
Classes (85)	77	79	2.6	3 (3.7)	5 (6.1)	74 (90.2)	82
Phyla (80)	41	42	2.4	3 (6.7)	4 (8.9)	38 (84.4)	45

The occurrence of taxonomic rank members in replicates from only healthy, healthy and diseased or only diseased samples is shown at various levels of taxonomic ranks.

Table 3.3: Order-level comparison of pooled 16SrDNA clone libraries from healthy (N = 4) and diseased (N = 4) *M. faveolata* samples

Taxonomic rank (Phylum; Class; Order)	Healthy (%)	Diseased (%)	P-value
Proteobacteria; Alphaproteobacteria; Rhodobacterales	19 (6.0)	89 (25.6)	1.05×10^{-11}
Proteobacteria; Epsilonproteobacteria; Campylobacterales	0 (0)	16 (4.6)	3.23×10^{-05}
Planctomycetes; Planctomycetacia; Planctomycetales	19 (6.0)	7 (2.0)	8.30×10^{-03}
Firmicutes; Clostridia; Clostridiales	2 (0.6)	10 (2.9)	3.38×10^{-03}

Taxa with significant differences ($P < 0.05$) between libraries from healthy and diseased tissues are shown with the total number and percentages (%) of clones in the respective libraries. The 16SrDNA sequences were classified and compared using the LIBCOMPARE tool of the Ribosomal Database Project II (Cole et al., 2007) at a confidence threshold of 95 %.

Differentially abundant bacterial families in healthy versus diseased coral samples

Analysis of pooled clone libraries (healthy: N = 317; diseased: N = 340) suggested a significant difference ($P < 0.05$) in the orders Rhodobacterales, Campylobacterales, Planctomycetales and Clostridiales (Table 3.3). PhyloChip hybridizations (healthy: N = 4; diseased N = 4) suggested that among a total of 275 families detected, 25 were found to contain at least one OTU that was significantly more abundant in samples from diseased corals (Table 3.4). Both methods agreed in finding the orders Rhodobacterales and Clostridiales as significantly more abundant in diseased samples, but an increase in Campylobacterales and a decrease in Planctomycetales as suggested by clone library data were not reflected in PhyloChip data. Although most of the sequences affiliated to Planctomycetaceae could not be classified at the genus level, all Campylobacterales belonged to the genus *Arcobacter*.

Classified 16S rDNA clone library sequences from healthy and diseased samples were mapped back to differentially abundant families and in addition compared to each other before and after filtering based on the PhyloChip results (Table 3.4). Twelve of the differentially abundant families contained at least one 16S rDNA representative among the combined clone libraries. With few exceptions, the significant increase in PhyloChip signal intensities was reflected in a greater number of 16S rDNA clones when libraries from healthy and diseased corals were compared (Table 3.4). Although three families (Alteromonadaceae, Enterobacteriaceae and Vibrionaceae) that are known to include coral pathogens were found to be more abundant in diseased coral samples, neither PhyloChip (similarity between assayed OTU and *Aurantimonas coralicida* is approximately 99.9 %; Supplementary Table S1) nor clone library data (N = 340 sequences from diseased libraries) provided any evidence for the presence of the WPD-II causing pathogen *A. coralicida*.

Similarity searches revealed that 4.1 and 18.2 % of clone libraries from healthy and diseased coral samples, respectively, matched to sequences that were previously identified in diseased, stressed or injured marine invertebrates (for details see Supplementary Table S2). After integration of our PhyloChip results,

Table 3.4: Differentially abundant bacterial families in healthy versus White Plague diseased *M. faveolata* samples are shown according to Phylochip G2 results (first column)

Differentially abundant families (Phylochip: > 2.0 fold change)	No. of clones healthy ($N_{total} = 317$)	No. of clones diseased ($N_{total} = 340$)
Actinobacteria; Actinomycetales; Cellulomonadaceae*	0	0
Actinobacteria; Actinomycetales; Corynebacteriaceae*	0	0
Actinobacteria; Actinomycetales; Micrococcaceae*	0	0
Actinobacteria; Actinomycetales; Unclassified*	0	1
Bacteroidetes; Bacteroidales; Bacteroidaceae*	0	0
Flavobacteria; Flavobacteriales; Flavobacteriaceae*	9	10
Bacilli; Bacillales; Bacillaceae*	1	0
Clostridia; Clostridiales; Clostridiaceae*	1	0
Clostridia; Clostridiales; Lachnospiraceae*	0	0
Clostridia; Clostridiales; Peptostreptococcaceae*	1	10
Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae**	18	89
Alphaproteobacteria; Rhodobacterales; Unclassified*	0	0
Betaproteobacteria; Burkholderiales; Comamonadaceae*	0	0
Betaproteobacteria; Hydrogenophilales; Hydrogenophilaceae*	0	0
Betaproteobacteria; Rhodocyclales; Rhodocyclaceae*	0	0
Gammaproteobacteria; Alteromonadales; Alteromonadaceae**	3	11
Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae**	0	0
Gammaproteobacteria; Alteromonadales; Shewanellaceae*	0	0
Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae*	0	0
Gammaproteobacteria; Oceanospirillales; Halomonadaceae*	1	0
Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae**	0	1
Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae*	0	2
Gammaproteobacteria; Thiotrichales; Piscirickettsiaceae*	0	0
Gammaproteobacteria; Unclassified; Unclassified**	1	3
Gammaproteobacteria; Vibrionales; Vibrionaceae**	3	6
<i>Vibrio vulnificus</i> st**	0	0
No of clones previously found in health-impaired ^a marine invertebrates/total	9/38 (23.7 %)	57/133 (42.9 %)

The number of 16S rDNA clones assigned to each family (second and third column) and the sum of clones with similarities to bacteria previously isolated from health-impaired marine invertebrates (last row) are shown. Families that include known coral pathogens are boldfaced.

^aStressed, diseased or injured.

*P < 0.01, **P < 0.001).

that is, if only 16S rDNA clones that were classified according to the differentially abundant families were considered, the proportion of sequences that were previously identified in diseased, stressed or injured organisms increased from 18.2 to 42.9 % (Table 3.4). Fifty-seven out of 62 clones (92 %) that were similar to bacteria associated with stressed, diseased or injured marine invertebrates were affiliated to the statistically more abundant families.

Differentially abundant family members

Among the bacteria that were affiliated with the significantly more abundant families, we found close relatives to the putative pathogen of the Pacific white syndrome in the Red Sea, *Thalassomonas loyana* (Thompson et al., 2006) and *Photobacterium eurosenbergii*, a *Vibrio*-like bacterium that has been associated with coral bleaching (Thompson et al., 2005). In addition, we found close relatives to bacteria that had been isolated from corals with Black Band Disease, including

members of Rhodobacteraceae that are known to associate with toxic dinoflagellates (Supplementary Table S2). Other 16S rDNA sequences that were classified to the family Peptostreptococcaceae had also been previously identified in Black Band Disease tissues (Supplementary Table S2).

Two 16S rDNA ribotypes had similarities to Rhodobacteraceae members that were found in body wall lesions of the sea urchin *Tripneustes gratilla*. Both of them were present in libraries from healthy as well as diseased corals. One of them (most similar to AM930419) was found only once in both libraries, whereas the other one (most similar to AM930434) was found two times in the library from healthy, but four times more often in the library from diseased coral samples (Supplementary Table S2). *Vibrio harveyi*-like (similarity > 99.7 %) bacteria, previously shown to be associated with pathogenesis in the turbot *Scophthalmus maximus*, were identified in both healthy and diseased coral samples and bacteria associated with skin ulceration disease and viscera ejection syndrome of the sea cucumber *Apostichopus japonicus* were exclusively found in samples from diseased corals (Supplementary Table S2).

There were also a number of other differentially abundant families that contained at least one classified 16S rDNA clone from healthy or diseased coral samples. These included Flavobacteriaceae, Bacillaceae, Clostridiaceae, Pseudomonadaceae, all of which include well-studied pathogenic members. According to PhyloChip data, there were additional families that were significantly increased in diseased coral samples for which no clone library support was available (Table 3.4). Finally, a probe set specifically designed to detect the marine pathogen *Vibrio vulnificus* suggested an increased abundance of this bacterium (or a close relative) in diseased coral samples (see Supplementary Information file).

3.5 Discussion

PhyloChip and clone library sequencing as a dual approach to study coral microbiology

The availability of universal 16S rRNA gene primers has made it possible to amplify a mixed population of 16S rDNA molecules and to characterize the phylogenetic diversity of coral-associated bacterial communities (Rohwer et al., 2002; Kellogg, 2004; Wegley et al., 2004; Bourne and Munn, 2005). Rohwer et al. (2002) estimated a richness of more than 6000 ribotypes in three different coral species based on 1178 sequenced 16S rDNA clones (Rohwer et al., 2002). Although species richness may be predicted from only a few hundred sequences, it has been suggested that identifying 50 % of a community of comparable composition would have required about 30000 sequencing reactions (Dunbar et al., 2002). Our rarefaction analysis on OTUs from a single *Montastraea faveolata* colony illustrates that a comprehensive description of the high bacterial diversity harbored in corals would easily be impeded by costs of sequencing at the required depth (Figure 3.1).

This constitutes a major problem, as entire coral populations are increasingly afflicted by disease events (Sutherland et al., 2004; Weil et al., 2006), so that a rapid increase in our knowledge on both the diversity of and changes in bacterial communities is of critical importance. In this study, we show that the application of the PhyloChip was suitable to (a) detect the presence of sequenced 16S rDNA clones and categorize more than 70 % at the family level, (b) distinguish healthy from diseased corals based on hybridization signal profiles and (c) enrich, after statistical analysis, 16S rDNA sequences from clone libraries with similarities to bacteria that were previously isolated from diseased/stressed marine invertebrates, including known pathogens.

Coral-associated bacteria detected by PhyloChip hybridization and clone library sequencing

The PhyloChip (G2) was able to detect the presence of every phylum and class that was identified in the corresponding clone library. The same concurrence was observed for nearly all orders, families and subfamilies. At the OTU level, however, there was no match between PhyloChip data and 16S rDNA sequencing results. Besides the novelty of sequences at the OTU level, the percentages of classifiable sequences at higher levels of taxonomic resolution indicates the existence of novel families, orders and classes that are harbored by corals (Table 3.1). Given that the classification was based on the availability of nearly full-length (≥ 1250 bp) 16S rDNA sequences as of March 2004 (DeSantis et al., 2006b), the high level of novelty was not surprising. Previous studies had also reported that coral-associated bacteria shared low sequence similarities (50 % of over 1000 sequences of ~ 500 bp shared < 93 % similarity) to public database entries (Rohwer et al., 2002). Furthermore, 16S rDNA sequences from coral samples have rarely been sequenced to a length exceeding 1250 bp; however, the availability of nearly full-length sequences will be critical to increase the diversity assayed by future versions of 16S microarrays.

Comparison of healthy and diseased coral tissue samples

In addition to the adverse effects of rapid climate change and ocean acidification (Hoegh-Guldberg et al., 2007), coral reefs are increasingly threatened by a number of diseases (Sutherland et al., 2004; Weil et al., 2006). In 1995, an unusually aggressive disease outbreak affected several different coral species in the Caribbean exhibiting similar signs, namely, a prominently sharp lesion line separating healthy looking tissues from white, tissue-devoid areas progressing at a rate of up to 2 cm per day (Richardson, 1998). The same signs were observed in 16 other coral species and coined Plague Type II (Richardson et al., 1998a). A bacterium isolated from *Dichocoenia stokesi*, later characterized as a novel species named *Aurantimonas coralicida* (Denner et al., 2003), was identified as the causative agent of White Plague Disease type II. Subsequently, other sources (Richardson et al., 2005; Weil et al., 2006; Rosenberg et al., 2007) emphasized the notion that *A. coralicida* was a

broad host-range pathogen causing White Plague-like signs in many different coral species in both the Pacific and Atlantic regions.

In this study, neither PhyloChip hybridizations nor 16S rDNA clone library sequencing indicated that *A. coralicida* was present in putatively White Plague type II-diseased *M. faveolata* colonies. Instead, we observed an increase in diversity in samples from diseased tissues (Table 3.2), whereas there was no overwhelming dominance by a single bacterial species, which appears to be an unexpected result for a primary infection. It has been argued that opposed to the idea of a primary infection, coral diseases may also result from unchecked growth of otherwise harmless bacteria in compromised hosts and/or due to changes in the environment, for example, increased temperatures (Harvell et al., 1999, 2007; Lesser et al., 2007). The identification of bacteria in healthy corals that were similar to known pathogens or bacteria that were previously isolated from diseased, stressed or injured marine invertebrates may point toward a role of latent, usually non-pathogenic commensals. We also identified many bacteria closely related or identical to pathogens of other marine invertebrates in diseased corals, which may suggest a role of exogenous opportunistic pathogens of broad hostrange. Nevertheless, it should be noted that we are not able to conclude from our results whether an increased population of different bacteria is the cause or rather the result of a disease. Alternatively, colonization by opportunistic pathogens or uncontrolled growth of commensals may have taken advantage of a compromised host immune system caused by a primary agent and/or unfavorable environmental conditions such as an increase in available nutrients or the preceding bleaching episode in 2005.

Bacterial family members with increased abundance in diseased coral samples

Bacterial families that we found at higher abundance in diseased coral samples and that had previously been associated with coral disease or bleaching included members of Vibrionaceae and Alteromonadaceae. *Vibrio* spp. have previously been reported to either cause or be associated with higher prevalence in a number of coral diseases (Kushmaro et al., 1996; Ben-Haim and Rosenberg, 2002; Cervino et al., 2004; Gil-Agudelo et al., 2006, 2007; Sussman et al., 2008). Furthermore, *Vibrio* spp. have been characterized as pathogens for a variety of other marine organisms and their zoonotic potential is well-known (Amaro and Biosca, 1996; Gonzalez et al., 2004; Thompson et al., 2004). As our data represent only a temporal snapshot, we are not able to determine how an increase in Vibrionaceae is related to the development of the disease. Interestingly, it has been reported that an increase in *Vibro* spp. both reduces the protective properties of beneficial commensals in coral surface mucus layers during a bleaching event (Ritchie, 2006) and also precedes visible signs of bleaching (Bourne et al., 2008).

The bacterium *Thalassomonas loyana* belongs to the order Alteromonadales and has been identified as a coral pathogen for a white plague-like disease in the Red Sea (Thompson et al., 2006). An increase of similar ribotypes in diseased samples in our study may be an indication for the presence of a closely related

species in Caribbean white plague-diseased corals. Other Alteromonadales that were detected in higher abundance included family members of Alteromonadaceae and Pseudoalteromonadaceae. Although we did not find support by clone library sequencing, it should be noted that some members of Pseudoalteromonadaceae have been shown to possess algicidal properties (Lovejoy et al., 1998; Ivanova and Mikhailov, 2001; Mayali and Azam, 2004), which were proposed to play a role in the causation of Yellow Blotch Disease (Cervino et al., 2004). The rapid progression of tissue whitening in white plague-like diseases could possibly be related to algicidal activities of bacteria that increase in abundance in diseased tissues.

A higher abundance of Rhodobacterales as indicated by PhyloChip results was highly consistent with the data obtained from clone library sequencing. The fact that many ribotypes belonging to Rhodobacterales were shared among healthy and diseased samples, but occurred at higher numbers in diseased tissues, could point towards an unchecked growth of opportunistic commensals as a response to disease. The significant increase in *Arcobacter* spp. (Supplementary Table S3) in diseased coral samples (only detected by clone library sequencing) is suggestive for a role of human and/or agricultural sewage in the development of the disease. The genus *Arcobacter* belongs to the Epsilonproteobacteria and comprises two species, *Arcobacter butzleri* and *Arcobacter cryaerophilus*, which can be found in animal livestock (Suarez et al., 1997; Wesley et al., 2000) and in association with human diarrheal illness (Kiehlbauch et al., 1991). *A. butzleri* is also a close taxonomic relative of known human pathogens such as *Campylobacter jejuni* and *Helicobacter pylori* (Miller et al., 2007). Other differentially abundant families included Flavobacteriaceae, Bacillaceae, Peptostreptococcaceae and Clostridiaceae, which include a multitude of examples of pathogenic species (Baron, 1996).

Conclusions and future outlook

Corals are simple organisms with limited phenotypic responses (disease signs), some of which may be similar for different infections. In this study, field collections were done after colonies recovered from intense and long-lasting thermal stress conditions, which presumably triggered the epizootic event observed in Puerto Rico and the Virgin Islands. It is possible that the bleaching event had already changed the microbiota, which may have contained bacteria that became pathogenic under repeated stressful conditions. Although we were not able to deduce more details about the etiology of the disease, we have shown that a combinatorial approach of PhyloChip hybridizations and clone library sequencing can substantiate a list of candidates that may play a significant role in disease development based on statistical support. Furthermore, the absence of *A. coralicida* suggest that White Plague Disease, in its current usage, refers to a group of distinct diseases with similar signs in different species, which necessitates further characterization at the pathological, cellular and molecular level at spatial and temporal scales.

With the advent of continued 16S rDNA sequencing efforts, it should be

soon possible to design and implement high-density microarrays with higher sensitivity and specificity for coral-associated bacteria. We have demonstrated the ability of this technology to distinguish bacterial profiles of healthy and White Plague-diseased *M. faveolata* colonies, so that future work should be dedicated to extend the number of coral species and diseases investigated. Furthermore, temporal changes in bacterial community structures before, during and after a disease episode necessitates detailed investigation to address the question of whether changes in particular bacterial populations are an indication for the cause or rather the result of a disease. Future studies may also validate the application of PhyloChips as a versatile platform for monitoring and assessing reef water quality. This technology could also be coupled with other methods to assess the health state of corals including biochemical assays (Downs et al., 2005) or gene expression microarrays (Morgan et al., 2005; DeSalvo et al., 2008). The generation of such data sets could guide the implementation of these technologies in effective management strategies to preserve the most diverse marine ecosystem: coral reefs.

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3.8 Supplementary information

Supplementary Information is available online at:

<http://www.nature.com/ismej/journal/v3/n5/supinfo/ismej2008131s1.html>

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4 Coral Transcriptome Linked to *Symbiodinium* Genotypes

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4.1 Abstract

A mutualistic relationship between reef-building corals and endosymbiotic dinoflagellates (*Symbiodinium* spp.) forms the basis for the existence of coral reefs. Genotyping tools for *Symbiodinium* spp. have added a new level of complexity to studies concerning cnidarian growth, nutrient acquisition, and stress. For example, the response of the coral holobiont to thermal stress is connected to the host-*Symbiodinium* genotypic combination, as different partnerships can have different bleaching susceptibilities. In this study, we monitored *Symbiodinium* physiological parameters and profiled the coral host transcriptional responses in acclimated, thermally stressed, and recovered fragments of the coral *Montastraea faveolata* using a custom cDNA gene expression microarray. Interestingly, gene expression was more similar among samples with the same *Symbiodinium* content rather than the same experimental condition. In order to discount for host-genotypic effects, we sampled fragments from a single colony of *M. faveolata* containing different symbiont types, and found that the host transcriptomic states grouped according to *Symbiodinium* genotype rather than thermal stress. As the first study that links coral host transcriptomic patterns to the clade content of their *Symbiodinium* community, our results provide a critical step to elucidating the molecular basis of the apparent variability seen among different coral-*Symbiodinium* partnerships.

4.2 Introduction

Reef-building corals are critically important to the functioning of tropical coral reefs, the most biologically diverse and complex marine ecosystems. A mutualistic partnership between coral hosts and photosynthetic dinoflagellates in the diverse genus *Symbiodinium* is a well-documented driving force of the trophic and structural integrity of coral reef ecosystems. By trapping solar energy and nutrients, *Symbiodinium* provide up to 95 % of the energy requirements of the coral hosts, which precipitate calcium carbonate skeletons at high rates (Muscatine, 1990).

In recent decades, coral mortality and extinction risk have increased dramatically, predominantly owing to mass bleaching events that have become more intense and more frequent (Carpenter et al., 2008; Hughes et al., 2003). The term bleaching describes the paling of the coral tissue due to a disruption of the symbiosis between coral hosts and their obligate dinoflagellate endosymbionts resulting in a loss of endosymbiont cells and/or their photosynthetic pigments. Although bleaching can be triggered by a number of factors, high temperature and light stress are commonly considered as the ecologically most significant (Hoegh-Guldberg, 1999). Under short or mild thermal stress conditions, corals may recover; although, adverse effects such as reduced growth and fecundity, as well as higher disease susceptibility, have been observed in subsequent periods (Bruno et al., 2007; Harvell et al., 2002; Sunagawa et al., 2009).

Our knowledge of the complexity underlying coral-dinoflagellate symbioses has increased with the advent of molecular genotyping tools for *Symbiodinium* spp. (reviewed by Baker, 2003; Coffroth and Santos, 2005; Pochon et al., 2006; Stat et al., 2006). Clade genotyping via restriction fragment length polymorphism (RFLP) of the 18S rRNA gene *Rowan:1991* and subclade genotyping via differential gradient gel electrophoresis (DGGE) of the internal transcribed spacer 2 (ITS2) region are now in common practice (LaJeunesse and Trench, 2000). Many coral species house only one clade (Goulet, 2006), some species house many different clades (Frade et al., 2008; Mieog et al., 2007; Rowan and Knowlton, 1995; Toller et al., 2001), and some species can shuffle their *Symbiodinium* clade content following bleaching events (Berkelmans and van Oppen, 2006; Jones et al., 2008; Rodriguez-Roman et al., 2006; Thornhill et al., 2006; Toller et al., 2001). The composition of coral-*Symbiodinium* partnerships has been shown to affect a number of important properties, including (1) adaptation to different environmental optima (Iglesias-Prieto et al., 2004; Rowan and Knowlton, 1995), (2) differences in growth rates (Little et al., 2004; Mieog et al., 2009), (3) photosynthate transfer and carbon fixation efficiencies (Cantin et al., 2009; Loram et al., 2007; Stat et al., 2008), and (4) susceptibility to thermal stress (Baker et al., 2004; Middlebrook et al., 2008; Mieog et al., 2009; Rowan et al., 1997; Sampayo et al., 2008).

Given the evidence that different symbiont genotypes can affect these processes, correlations between the *Symbiodinium* clade content and coral gene ex-

pression have the potential to inform us on the molecular basis of differences in coral-*Symbiodinium* partnerships. Gene expression microarrays have been widely used to investigate changes in the expression of all or a subset of transcribed genes (i.e. the transcriptome) under varying conditions. Gene expression differences in cnidarian holobionts have thus far been studied in symbiotic versus aposymbiotic anemones (Rodriguez-Lanetty et al., 2006), under thermal and UV stress (DeSalvo et al., 2008; Richier et al., 2008), during acclimatization of different source populations to different environmental conditions (Bay et al., 2009), during the onset of symbiosis (Voolstra et al., 2009), and across distinct developmental stages (Grasso et al., 2008; Reyes-Bermudez et al., 2009).

In this study, we measured *Symbiodinium* physiological parameters and profiled host gene expression in acclimated, thermally stressed, and recovered fragments from different colonies of *Montastraea faveolata* a coral known to associate with multiple clades of *Symbiodinium* (Iglesias-Prieto et al., 2004; Rowan and Knowlton, 1995; Thornhill et al., 2006; Toller et al., 2001). In a parallel experiment, fragments harboring different *Symbiodinium* clades were sampled from a single host colony in order to control for the host genotypic background. Based on photochemical efficiency and host gene expression measurements in fragments containing different *Symbiodinium* clades, we demonstrate that the coral host transcriptional state, as well as the magnitude of the thermal stress response, is correlated with the symbiont genotype.

4.3 Materials and methods

Sample Collection and Tank Experiment

On 31 July 2007, six replicate fragments were collected using a hammer and chisel from the top sun-exposed surface of five different, healthy-looking *Montastraea faveolata* colonies near Puerto Morelos, Quintana Roo, México (20°52'28.77" N and 86°51'04.53" W). These 30 fragments were used in a time-series experiment of control, thermal stress, and recovery (referred to as the "time-series experiment"). In addition, six replicate fragments were sampled from the top (2.7 m), middle (3.7 m), and bottom (5.2 m) of a single colony of *M. faveolata*. These 18 fragments were used in a single time point experiment of control vs. thermally stressed (referred to as the "single host genotype experiment"). All fragments were transported to the UNAM - Instituto de Ciencias del Mar y Limnología field station within 1 h and divided evenly between two 50 L aquaria (i.e., 3 fragments from each colony and 3 fragments from top, middle, and bottom parts of one single colony were placed into each tank) that received a constant flow of seawater (0.6 L min⁻¹) from the same source.

Acclimation of collected coral fragments

Control and experimental aquaria were placed in a large common fiberglass

tank with constantly flowing water to equilibrate the temperature of incoming seawater and to buffer temperature fluctuations. Both aquaria were exposed to the same shaded ambient light condition in order to control for the light-related factors that determine the ecological zonation of *Symbiodinium* spp. within *M. faveolata*. Each aquarium was fit with a water pump connected to a spray-bar to provide constant water movement and aeration. All coral fragments were mounted on plasticene and kept at a depth of 7 cm. From 10 to 19 August 2007, both aquaria received an average water temperature of $27.9 \pm 0.6^\circ\text{C}$ (as recorded by HOBO Light/Temperature Data Loggers, Onset Corp.). Beginning on 11 August, dark-adapted maximum quantum yields for charge separation (Fv/Fm) were measured at dusk for all coral fragments using a DIVING-Pulse Amplitude Modulated (PAM) fluorometer (Walz, Germany). Photosynthetically active radiation (PAR) was measured at noon and averaged $318 \pm 129 \mu\text{mol m}^{-2} \text{s}^{-1}$. From 20 to 21 August 2007, all coral fragments were brought inside during the passage of Hurricane Dean. On 22 August, the experiment was reconstituted.

During acclimation of the coral fragments, top fragments in the single host genotype experiment did not show any significant variation in Fv/Fm (0.575 ± 0.030) relative to the values observed in the field (0.550 ± 0.028). Likewise, none of the 30 fragments in the time-series experiment displayed a significant decrease in Fv/Fm throughout the acclimation period. In contrast, middle fragments in the single host genotype experiment experienced a significant reduction in Fv/Fm upon transfer to the experimental tanks from 0.582 ± 0.023 in the field to 0.481 ± 0.054 after 48 hours in the tanks. Similarly, bottom fragments experienced a dramatic reduction of Fv/Fm after transfer to the experimental tanks from 0.610 ± 0.030 in the field to 0.348 ± 0.042 after 48 hours of exposure to the experimental tank conditions. However, at the end of the control period (see below), all coral fragments were completely acclimated to the experimental tank conditions (Figure S1).

The control period of the experiments started on 23 August (day 1) and lasted until 1 September (day 10). During this time, both aquaria received a mean water temperature of $28.5 \pm 0.8^\circ\text{C}$, and mean PAR of $371 \pm 169 \mu\text{mol m}^{-2} \text{s}^{-1}$. On the night of day 1, control time point samples from five different colonies were collected from each tank. Afterward, one 200-Watt aquarium heater was turned on in the treatment aquarium (rate: $0.35^\circ\text{C h}^{-1}$). A second heater was turned on 3 days later. During the thermal stress period, the control aquarium received mean water temperature of $28.8 \pm 1.2^\circ\text{C}$, the heated aquarium $31.5 \pm 1.1^\circ\text{C}$, and both tanks received mean PAR of $420 \pm 152 \mu\text{mol m}^{-2} \text{s}^{-1}$. On the night of day 16, all fragments in the single host genotype experiment were flash-frozen in liquid nitrogen. On day 20, and day 58, one sample each from the five colonies used in the time-series experiment was collected from both tanks for the thermal stress and recovery time point, respectively. All samples were exported to the USA through a CITES permit (MX-HR-007-MEX). See SI Text for methods related to statistical analysis of Fv/Fm data.

Symbiodinium and Coral Host Genotyping

Genomic DNA was isolated from approximately 100-200 mg of frozen coral powder using the PowerPlant DNA Isolation kit (MoBio). The *Symbiodinium* 18S ribosomal RNA gene was amplified using the primers ss5 and ss3Z (Rowan and Powers, 1991) and digested with TaqI restriction enzyme. The resulting fragments were compared to *Symbiodinium* clade standards (Rowan and Knowlton, 1995). The internal transcribed spacer region 2 (ITS2) was PCR amplified using cycling conditions and primers ITSintfor2 and ITS2CLAMP (without the clamp sequence) reported in (LaJeunesse and Trench, 2000). ITS2 amplicons were cloned, sequenced, and assigned to *Symbiodinium* clades based on both BLASTn results and phylogenetic reconstruction using reference sequences (see SI Text for details). All ITS2 sequences were deposited in GenBank with the accession numbers FJ223886-FJ224080 and FJ811907-FJ811960. Coral hosts were genotyped (see SI Text for details) using five microsatellite loci (Severance et al., 2004).

Microarray Gene Expression Analysis of Coral Host Gene Expression

Total RNA from all samples was isolated and checked for quality as previously described (DeSalvo et al., 2008). Contamination of total RNA with *Symbiodinium* RNA is expected to be low as determined by microscopic analysis of re-suspended coral powder, which revealed *Symbiodinium* cells to be intact. For the time-series experiment, a pool of amplified RNA from all control tank fragments was used as a reference RNA sample, against which each of the 15 amplified RNA samples from the treatment tank (5 control, 5 stressed, 5 recovered) was competitively hybridized. For the single genotype experiment, the reference sample constituted a pool of total RNA from all 18 samples. Fifteen micrograms of total RNA from control (3 replicates per region) and heat-stressed (3 replicates per region) samples were competitively hybridized against an equal amount of reference RNA resulting in a total of 18 hybridizations. Dye swaps were not performed, as any dye bias present is equal in all comparisons to the reference.

Samples were hybridized to *M. faveolata* microarrays containing 2620 features (1310 genes spotted in duplicate) that were annotated based on BLASTx hits (E-value cutoff: $1e^{-5}$) to the UniProt Knowledgebase database SwissProt and its GO-term associations (UniProt GOA, March 2008). All sequences/clone IDs are searchable at: <http://sequoia.ucmerced.edu/SymBioSys/index.php>. Based on hybridization of *Symbiodinium* RNA directly to the *M. faveolata* microarray, we determined the potential for cross-hybridization with *Symbiodinium* transcripts to be below 8 % (data not shown). All microarrays were scanned using an Axon 4000B scanner (Molecular Devices) where care was taken to manually balance photomultiplier tube (PMT) settings. TIFF images were generated with GenePix Pro 6.0, and gridding was performed using TIGR Spotfinder 3.1.1 (Saeed et al., 2003) with the Otsu segmentation method. The top 25 % of background pixels were discarded prior to the estimation of the median local background intensity, which was sub-

sequently subtracted from the median foreground intensity. Using TIGR MIDAS 2.19 (Saeed et al., 2003), background-corrected data were LOWESS normalized, and in-slide duplicates were averaged. Both PMT balancing and LOWESS normalization equalize for differing amounts of host RNA input (a potential issue when processing tissue where symbiont densities can vary). Genes were included in statistical analyses only if at least 60 % of representative spots were called positive in each condition tested. This corresponds to three out of five (time course experiment), or two out of three (single host genotype experiment) hybridizations for a given category (i.e., control, thermal stress, recovery, or top-control, top-treatment, etc., respectively). After this filtration step, 1012 (time-series experiment) and 1236 (single host genotype experiment) genes were used for subsequent analyses. Microarray hybridization data (both raw and normalized) along with methodological details are deposited in GEO with the series record number GSE15262.

The ratio between the fluorescence intensity of the two channels was used as input for BAGEL (Bayesian Analysis of Gene Expression Levels) (Townsend and Hartl, 2002). The BAGEL software uses Bayesian probability to infer a relative expression level of each gene. An estimated mean and 95 % credible interval of the relative level of expression of each gene is computed in each treatment and time point. We used the conservative gene-by-gene criterion of non-overlapping 95 % credible intervals to regard a gene as significantly differentially expressed. The following pair-wise statistical tests were performed: control>stressed, control<stressed, control>recovered, control<recovered, stressed>recovered, and stressed<recovered (time-series experiment) and control>stressed, control<stressed (single genotype experiment). Similarly, gene expression in top (T), middle (M), and bottom (B) fragments under both control and stressed conditions were tested: T>M, T<M, T>B, T<B, M>B, and M<B. The genes found to be differentially expressed under these conditions were further grouped into 12 possible gene expression patterns, i.e.: B>M>T, B<M<T, B=M>T, B=M<T, B>M=T, B<M=T, B=T>M, B=T<M, B>M<T and B>T, B<M>T and B<T, B>M<T and B<T, and B<M>T and B>T.

Hierarchical clustering of gene expression was performed using TIGR TMEV 4.0 (Saeed et al., 2003). Array trees were created according to average linkage and Euclidean distance metric on \log_2 ratios of signal intensities, which were normalized across arrays in the case of the time-series experiment. Signal intensities from control and stressed replicate fragments ($n = 3$) from top, middle, and bottom were averaged prior to hierarchical clustering. The stability of clusters was tested using the R-package pvclust by calculating approximately unbiased p-values via multiscale bootstrap resampling; a measure that has been demonstrated to provide a better indicator for cluster support than regular bootstrapping procedures (Suzuki and Shimodaira, 2006). To assess over-representation of Gene Ontology (GO) terms in the lists of significant genes, we used default statistical tests and multiple-testing adjustments in GOEAST (Zheng and Wang, 2008) except the significance cutoff was set to $\alpha = 0.001$.

4.4 Results

Experimental Thermal Stress and Recovery

We measured dark-adapted maximum quantum yields for charge separation (Fv/Fm) in fragments sampled from five biological replicates of the coral *M. faveolata* throughout the time course of a thermal stress experiment (Figure 4.1A, Table S1). A significant decrease in Fv/Fm in previously acclimated coral fragments was observed four days after increasing the water temperature. The total duration of the thermal stress treatment was 10 days and the difference in Fv/Fm became insignificant two days after termination of the treatment (Figure 4.1A). The initial decrease in Fv/Fm was linear, whereas its increase during the recovery period was explained best by a logarithmic function (Figure S2, Table S2).

Differences in Fv/Fm among the sampled colonies were observed in all experimental periods ($p_{adj} < 0.001$; Table S1). Post-hoc analyses revealed that Fv/Fm averages in colonies C1 and C2 were significantly different from those in colonies C3, C4, and C5 during the control period. Colony C2 showed very little reduction in Fv/Fm upon heat treatment, and Fv/Fm values were significantly higher than in all other colonies during the stress and recovery periods. The differences between colonies were most likely due to a combinatorial effect of differences in *Symbiodinium* (Table 4.1) and host genotypes (Figure S3).

Restriction fragment length polymorphism (RFLP) analysis and sequencing of internal transcribed spacer 2 (ITS2) loci were used to genotype the *Symbiodinium* clades hosted by the experimental coral fragments. In the control samples, the dominant *Symbiodinium* clades were clade A in colonies 1 and 2, clade B in colonies 4 and 5, and clade C in colony 3. In thermally stressed samples, clade A was the dominant type in colony 3, whereas clade A and clade B remained the dominant clade types in colonies 1 and 2, and colonies 4 and 5, respectively (Table 4.1). While the 5 replicate colonies initially harbored a range of symbiont clades (A, B, and C), following recovery from bleaching all colonies harbored clade A, i.e., the dominant *Symbiodinium* clade type residing in colonies 3, 4 and 5 changed from clade C/B to clade A. ITS2 sequencing results were generally congruent with RFLP analyses in control fragments, and additionally allowed for both the determination of relative contributions of different *Symbiodinium* subtypes in fragments hosting more than one clade type and an increase in resolution of *Symbiodinium* clades to the subclade level. Based on both BLASTn results and phylogenetic reconstruction (Figure S4), we found the dominant haplotype of clade A, clade B, and clade C ITS2 sequences to be type A3 (most similar to EU074857), type B1 (EU074875), and type C7 (AF499797), respectively.

Analysis of a Single Host Genotype Harboring Different Symbiodinium Clades

In a parallel experiment, we sampled replicate fragments from top, middle and bottom parts of a single coral colony to obtain samples containing different *Symbiodinium* clade types in one host genetic background. Fv/Fm values began

Table 4.1: Results of *Symbiodinium* genotyping assays. The top half corresponds to the time series experiment, the bottom half to the single host colony experiment. When more than one sample appears on the same row, they contained the same *Symbiodinium* type and proportions. Abbreviations used: C, B, R followed by a number denote control, bleached, and recovered fragments, respectively.

Fragment(s)	18S rRNA RFLP		ITS2 sequencing				n
	1°	2°	1°	%	2°	%	
C1	A	-	A3	73	C7	27	11
C2	A	-	A3	90	C7	10	10
C3	C	B	C7	82	B1	18	11
C4	B	-	B1	91	C7	9	11
C5	B	-	B1	100	-	-	11
B1-3	A	-	n/a	-	n/a	-	-
B4	B	A	n/a	-	n/a	-	-
B5	B	-	n/a	-	n/a	-	-
R1-5	A	-	n/a	-	n/a	-	-
Top 1,3,6	B	-	B17	100	-	-	11,11,12
Top 2	B	C	B17	83	C7	17	12
Top 4	B	C	B17	80	C7	20	10
Top 5	B	C	B17	73	C7	27	11
Middle 1,4	C	-	C7	100	-	-	14,15
Middle 2	C	-	C7	69	B17	31	13
Middle 3	C	-	C7	82	B17	18	11
Middle 5	C	B	B17	75	C7	25	12
Middle 6	C	-	C7	67	B17	33	12
Bottom 1-6	C	-	C7	100	-	-	8-9

to decline following exposure to elevated temperature (Figure 4.1B). The bottom fragments showed a significant decrease relative to their controls two days after heating. Top fragments also showed a significant decrease after two days albeit less than the bottom fragments. The middle fragments showed a significant decrease after four days. By the end of the experiment, all heat-stressed fragments had significantly lower Fv/Fm values compared to their controls, and the top and bottom heat-stressed fragments had significantly lower maximum quantum yields than the middle heat-stressed fragments (Figure 4.1B). RFLP analyses revealed that middle and bottom fragments harbored mainly clade C; top fragments, mainly clade B (Table 4.1). Again, ITS2 sequencing results were largely consistent with RFLPs analyses (Table 4.1). After sequence alignment and phylogenetic analyses (Figure S4), we found that the dominant haplotype of clade C sequences was identical to type C7 (AF499797), and the dominant haplotype of clade B sequences was identical to type B17 (EU449083).

Microarray Gene Expression Analysis

After the generation of *Symbiodinium* physiological and genotypic data, we were interested in differentially expressed genes in control, thermally stressed, and recovered coral fragments from different coral colonies (Figure 4.2A). In addition, control and thermally stressed coral fragments from different regions of a single host colony were analyzed for differentially expressed genes (Figure 4.2B), a design that eliminated potential differences due to host genotypic background effects.

Pair-wise comparisons between control, stressed, and recovered samples resulted in a relatively small number ($n = 57-84$) of differentially expressed genes, which is most likely attributable to the different *Symbiodinium* genotypes that were hosted by the control and stressed fragments (Table 4.1). Nevertheless, a gene ontology (GO) enrichment analysis revealed that genes related to regulation of transcription and response to stress were significantly enriched ($p < 0.001$) among the annotated, differentially expressed genes (Figure fig:pub3fig2A, Table S3). A direct comparison between control and thermally stressed samples from a single host colony yielded only 28 differentially expressed genes with low fold-changes ranging from 1.08 to 1.53 (Figure 4.2B, Table S3).

Next, we performed hierarchical cluster analyses (using Euclidean distance) on all assayed (1000+) genes to group together samples with similar transcriptomic activity, and mapped *Symbiodinium* physiological and genotypic data onto the resulting array trees (Figure 4.3). With respect to the time-series experiment, the array tree reveals four major groups, which displayed congruency with *Symbiodinium* clade content and possibly with host genotypic differences. All recovered samples, which exclusively harbored *Symbiodinium* clade A, clustered together (Group 1, Figure 4.3A). In contrast, neither control nor thermally stressed samples were grouped together, i.e. according to experimental conditions. Instead, they were divided into groups that suggest the influence of both host and *Symbiodinium* genotype effects. Control and stressed samples from colony 2, which was

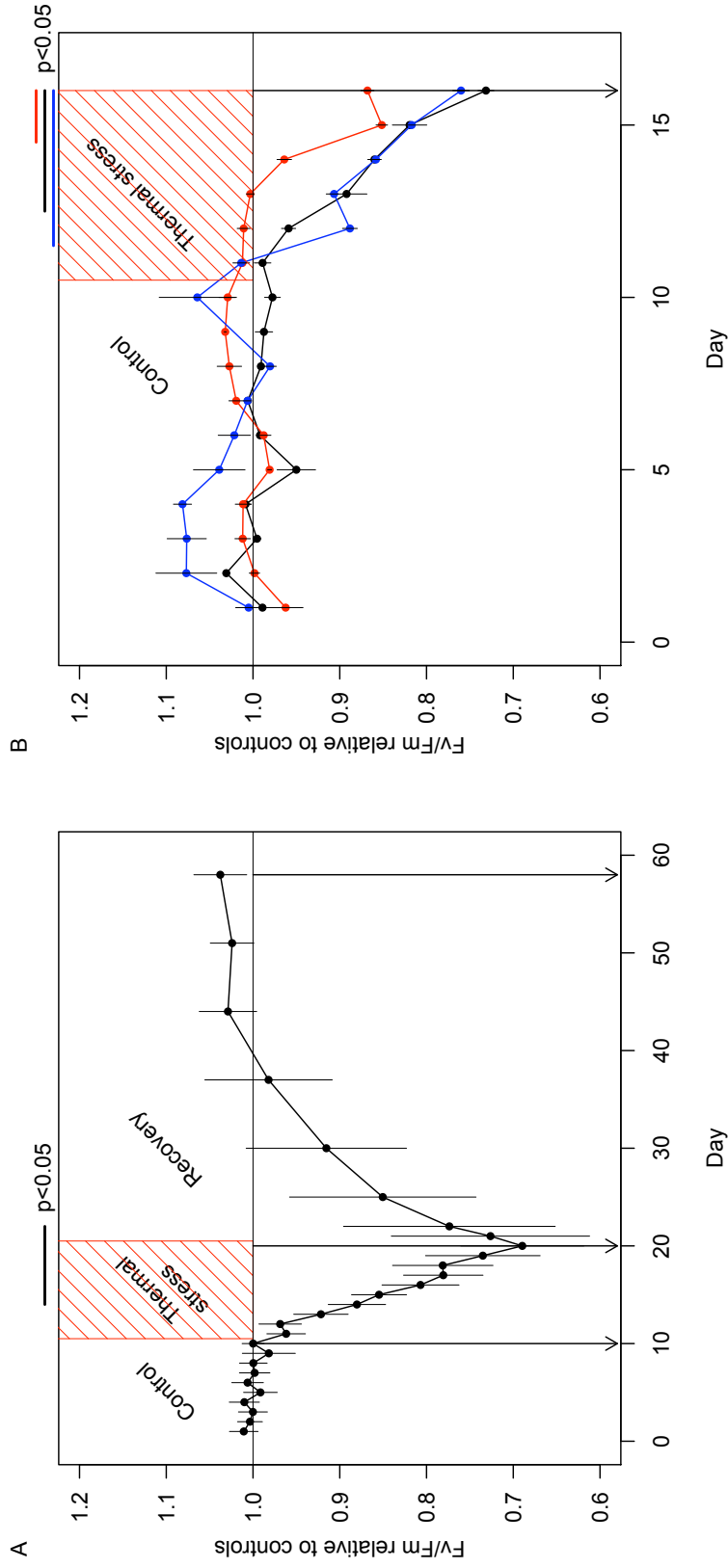


Figure 4.1: Relative F_v/F_m changes before, during, and after exposure of coral fragments to thermal stress. Dark-adapted maximum quantum yields relative to control samples were measured in five biological replicates during an experimental heat stress experiment (A) and in top (black), middle (red), and bottom fragments (blue) collected from a single coral colony (B). After acclimation, experimental coral fragments were exposed to elevated temperatures (shown by shaded areas) before being collected for microarray analyses at time points indicated by arrows. F_v/F_m data are averages (\pm standard error) of treatment tank replicates relative to the average of coral fragments maintained at control temperature. Horizontal bars above the plot indicate periods during which the difference between control and heat-stressed fragments were significant ($p < 0.05$).

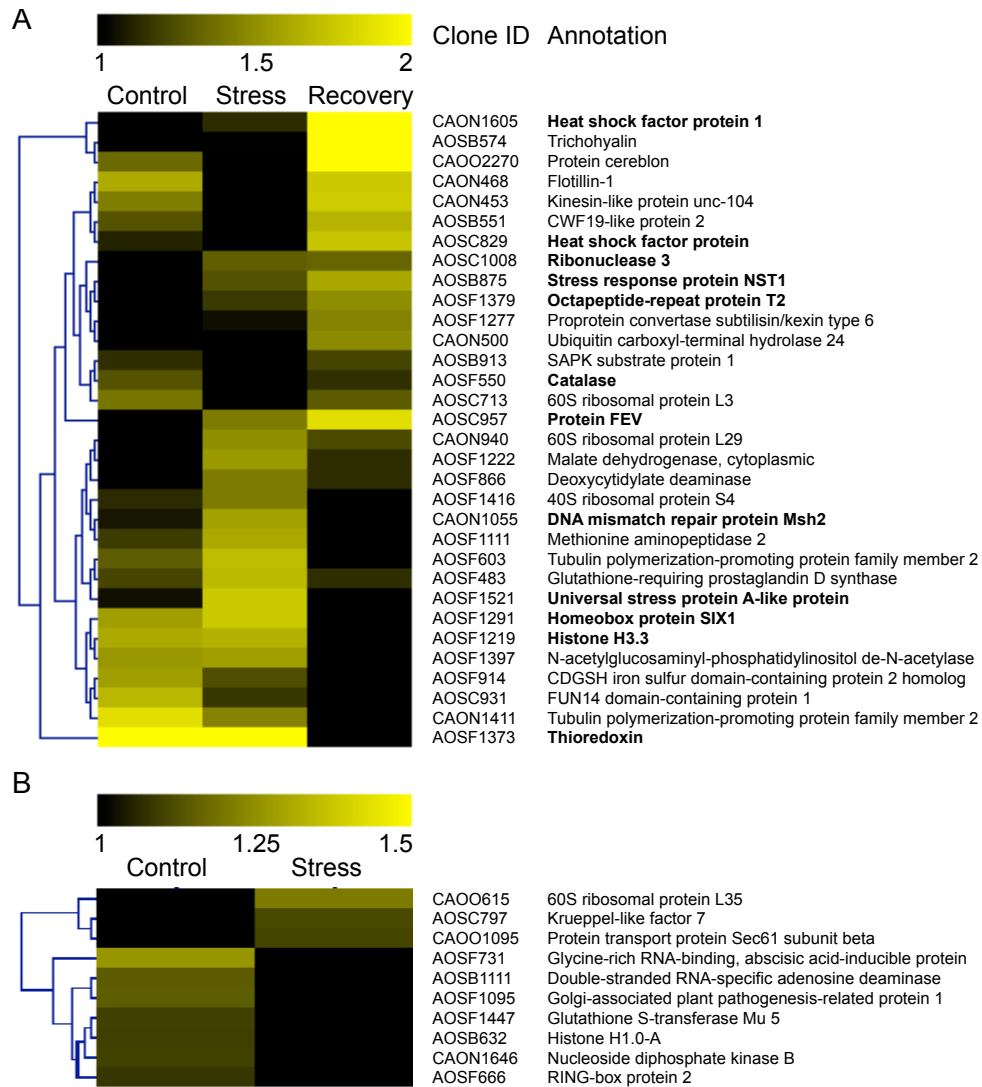


Figure 4.2: Heat map of stress-responsive differentially expressed genes. Annotated differentially expressed genes identified in the time-series (A) and single host genotype experiment (B) are shown with clone IDs and annotations. Color scales correspond to BAGEL-computed gene expression estimates where a value of 1 is assigned to the class with the lowest expression. Boldfaced genes are involved in transcription regulation and stress response according to GO enrichment analysis.

determined to be genetically distinct from all other colonies (Figure S3), contained mainly symbionts of clade A (Group 2, Figure 4.3A). Group 3 was formed by control and stressed fragments from colonies 1 and 3, which mainly contained clade A symbionts. The only exception to this observation was that the control fragment of colony 3 was dominated by clade C. These two colonies were genetically different from each other as well as different from colonies 2, 4 and 5 (Figure S3). Finally, different to all other control and stressed fragments, colonies 4 and 5 could not be genetically differentiated (Figure S3) and contained mainly clade B (Group 4, Figure 4.3A).

Another aspect of our analyses revealed an apparent trend between the magnitude of the transcriptomic response to thermal stress (determined by the distance between the control and stressed samples in Figure 4.3) and the symbiont genotype. In clade A (colonies 1 and 2) or clade C (colony 3) hosting fragments, the difference was relatively small (branch length distances between control and stressed samples: 37.02, 36.08, and 31.56, respectively) compared to those dominated by clade B (44.55 and 61.86 for colonies 4 and 5, respectively).

Unlike in the time-series experiment, differences in gene expression between samples originating from a single host genotype were not biased by host genotypic background, thus strengthening the observed correlation between host transcriptomic states and *Symbiodinium* clade contents (Figure 4.3B). We found that middle and bottom control fragments (both dominated by clade C symbionts) exhibited gene expression patterns more similar to each other than to clade B-dominated top fragments (Figure 4.3B top panel). This association was highly significant according to cluster support. However, the correlation between host transcriptomic states and *Symbiodinium* clade contents noticeably diminished in the top, middle, and bottom stressed fragments (Figure 4.3B lower panel). Not only were the transcriptomic states of the top and bottom stressed fragments more similar to each other than to the middle stressed fragments, but the branch lengths and cluster support were lower compared to the control tree (Figure 4.3B top panel). This pattern is consistent with the PAM data showing that *Symbiodinium* within top and bottom fragments experienced more stress than those within middle fragments (Figure 4.1B).

Expression Patterns of Differentially Expressed Genes

Testing for pair-wise differences between colony locations showed that among the control samples, many genes were differentially expressed between top and middle ($n = 204$) and top and bottom ($n = 312$); however, a very small number of genes were differentially expressed between middle and bottom ($n = 24$). These numbers were substantially lower for the corresponding analysis of thermally stressed samples: top vs. middle ($n = 51$); top vs. bottom ($n = 96$); and middle vs. bottom ($n = 49$) (see Table S3 for all gene expression results).

We further analyzed differentially expressed genes between top, middle, and bottom samples by grouping them into 12 major expression patterns. Among con-

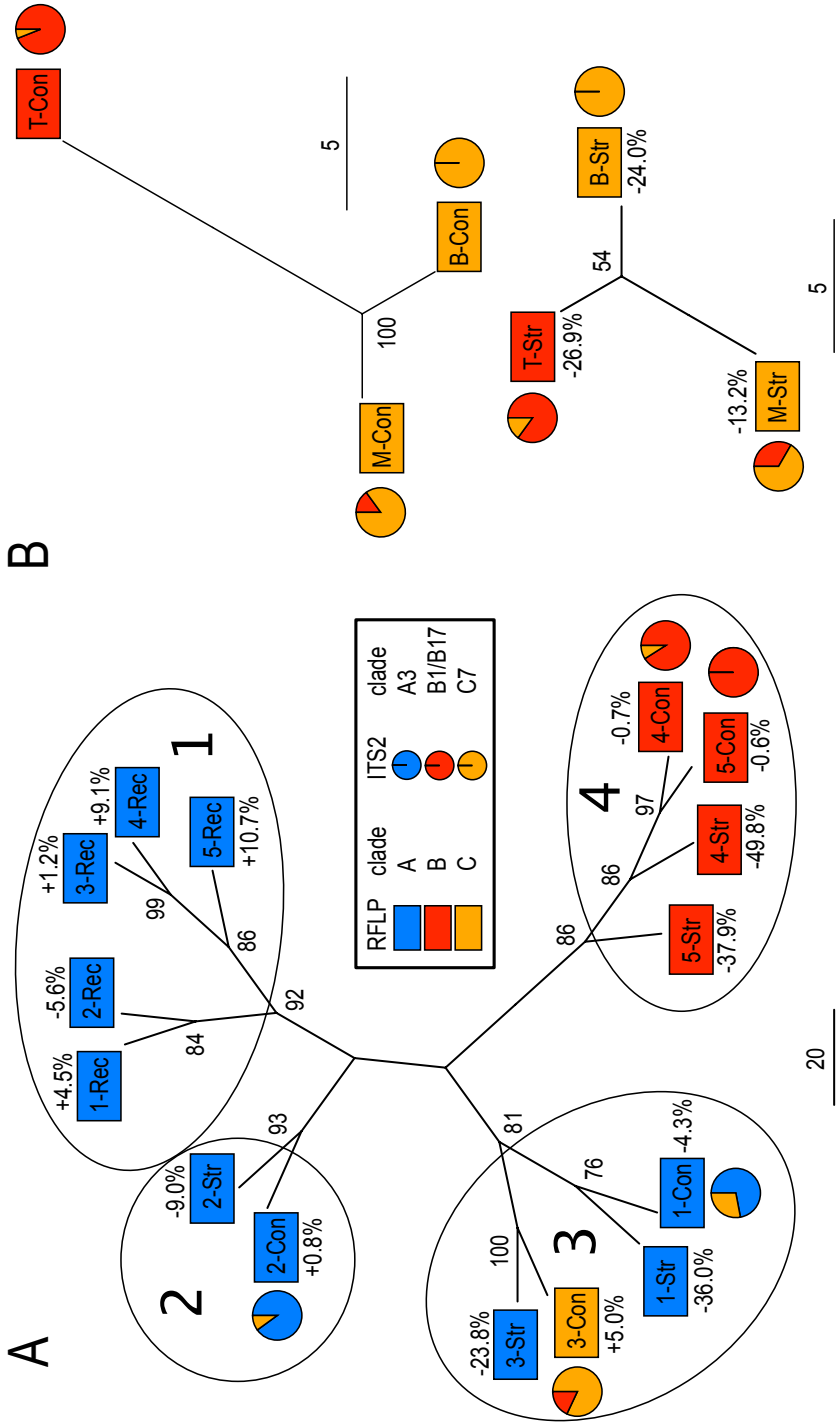


Figure 4.3: Hierarchical clustering of gene expression intensities shows that samples group according to differences in *Symbiodinium* clade content. (A) Samples from a thermal stress and recovery experiment using replicates from five different coral colonies were clustered into the following groups: (1) all recovered fragments, containing exclusively *Symbiodinium* clade A, (2) fragments from colony 2 containing predominantly clade A, (3) fragments from colonies 1 and 3 containing mainly clade A, and (4) fragments from colonies 4 and 5 containing predominantly clade B. (B) Averaged samples ($n = 3$) from top, middle, and bottom parts of a single coral host colony group according to *Symbiodinium* genotypes in control (top panel), but not thermally stressed samples (bottom panel). Legend: sample names (1-5: sampled colony; T: top, M: middle, B: bottom; Con: control, Str: thermally stressed, Rec: recovered) and RFLP analysis results are shown as color-coded rectangles. Pie charts illustrate proportions of *Symbiodinium* clades based on ITS2 sequence analysis in selected fragments (note: red indicates type B1 in panel A and type B17 in panel B). Numbers next to rectangles represent the percent differences in F_v/F_m between treated and untreated fragments. Approximately unbiased p-values are shown next to tree nodes.

trol samples, 173 genes were sorted into 8 different patterns. The vast majority (90 %) of these genes were either upregulated (B=M<T, n = 105) or downregulated (B=M>T, n = 50) in top versus middle/bottom samples (Figure 4.4, Table S3). The distribution into distinct patterns for stressed samples was more even, further supporting a diminishing correlation between transcriptomic states and *Symbiodinium* genotypes during thermal stress as previously mentioned (Figure 4.3B). Nevertheless, similar to the control samples, the majority (60 %) of differentially expressed genes were either upregulated (B=M<T, n=20) or downregulated (B=M>T, n = 6) in top versus middle/bottom samples (Figure 4.4, Table S3).

GO enrichment analysis of the most populated patterns, i.e. those with *Symbiodinium* genotype differences among the control samples (B=M<T and B = M>T), showed genes involved in protein metabolism (e.g., translation, protein folding/degradation) to be significantly ($p < 0.001$) enriched (Figure 4.5).

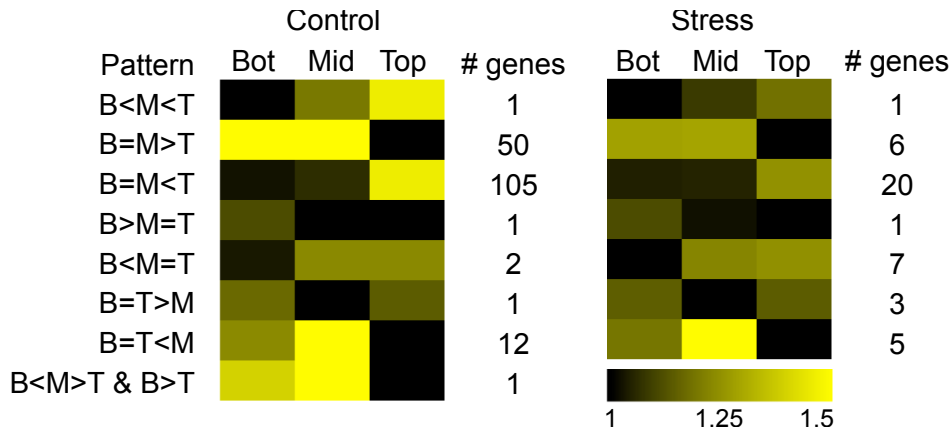


Figure 4.4: Pattern analysis of differentially expressed genes. Genes that were differentially expressed between top, middle, and bottom control (left panel) and stressed (right panel) fragments were sorted into groups of distinct expression patterns. Heat map intensities were calculated by averaging the BAGEL-estimated expression levels for all genes within each pattern. Of the 12 patterns tested, only those containing at least one gene are depicted.

4.5 Discussion

Host and Symbiodinium Factors Affect Dark-Adapted Maximum Quantum Yields (Fv/Fm)

Maximum quantum efficiencies in *Symbiodinium* sp. type C7-associated with *Montastraea faveolata* were previously shown to decrease more in response to experimental thermal stress compared to type B1-associated with *M. faveolata* (Warner et al., 2006). In our study, however, type B1-hosting colonies actually

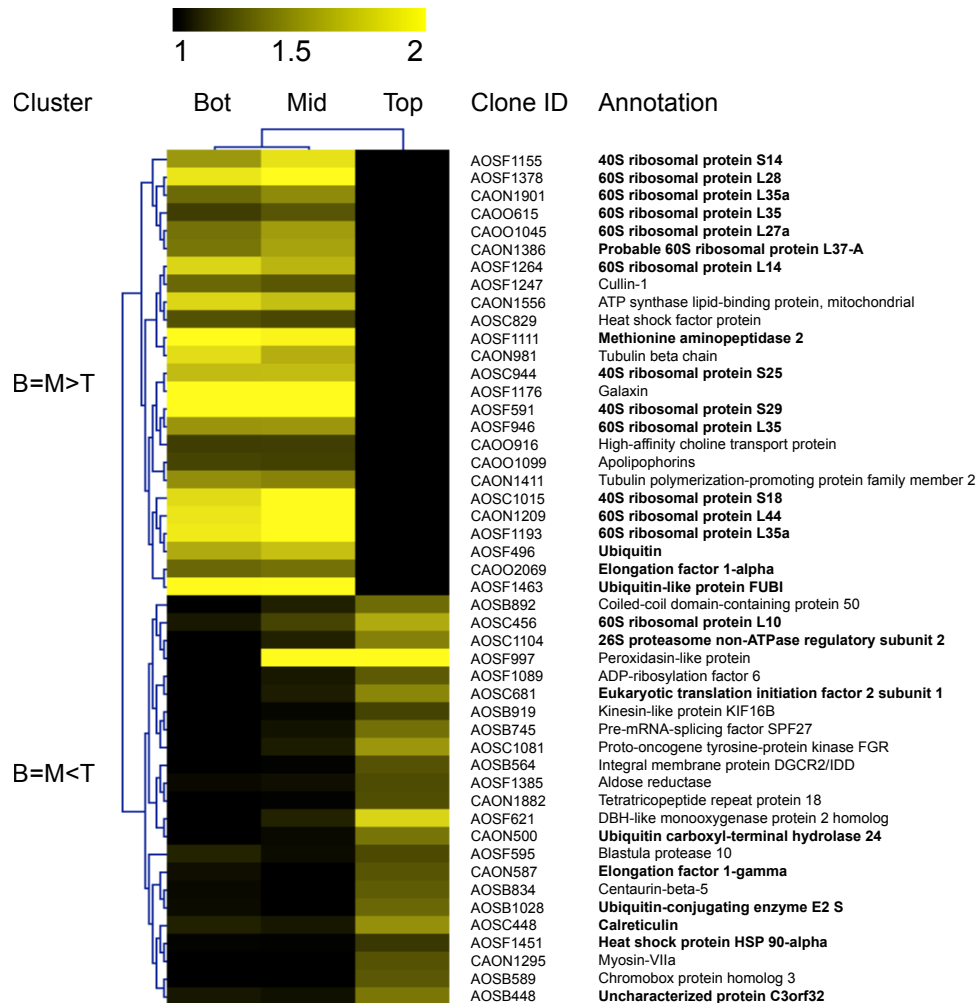


Figure 4.5: Heat map of genes differentially expressed between top, middle, and bottom control fragments. Annotated differentially expressed genes identified in the single host genotype experiment are shown with clone IDs and annotations. Color scales correspond to BAGEL-computed gene expression estimates where a value of 1 is assigned to the class with the lowest expression. Boldfaced genes are involved in protein metabolism (e.g., translation, protein folding/degradation) according to GO enrichment analysis.

decreased more in Fv/Fm than type A3- and type C7-containing colonies (Figure 4.3A). Similarly, type B17-containing top fragments experienced a greater decrease in Fv/Fm than type C7-containing middle fragments. However, the response of B17-containing top fragments was similar to C7-containing bottom fragments. Marked differences were also found in the degree of photoinactivation of PSII between different colonies that hosted the same *Symbiodinium* type. For example, colony 2 experienced hardly any reduction in Fv/Fm while colony 1 experienced a sharp decrease in Fv/Fm , yet both housed clade A.

The discrepancies between our study and Warner et al. (2006), in addition to the differences between middle and bottom fragments and the fact that colonies 1 and 2 differed in thermal susceptibility, argues against the idea that thermal stress-susceptibility of corals is solely linked to symbiont genotype (Iglesias-Prieto et al., 2004; LaJeunesse et al., 2007; Rowan, 2004; Sampayo et al., 2008; Tchernov et al., 2004). Instead, our results and other studies (Bhagooli and Hidaka, 2003; Brown et al., 2002; Goulet et al., 2005) support the notion that host contributions and/or effects of previous conditions (Brown et al., 2002; Middlebrook et al., 2008) must be taken into account when discussing differences in stress-induced Fv/Fm losses with respect to differences in *Symbiodinium* type.

Host gene expression profiling during thermal stress and recovery

In a previous coral microarray study on heat stress, we assayed gene expression changes in fragments from a single colony and fragments from different colonies (DeSalvo et al., 2008). We found that while using biological replicates (i.e., different colonies) increased our ability to draw conclusions beyond the limits of a single genotype, it confounded our ability to detect significant changes in genes that show only a small difference in expression given a similar number of microarrays. Nevertheless, many genes identified in the single-genotype experiment were corroborated in the multi-colony experiment (DeSalvo et al., 2008). In the present study, we followed a similar two-pronged experimental design by using multiple replicates from a single host colony, in combination with sampling different colonies.

Unexpectedly, we found: (1) few genes differentially expressed in response to thermal stress and recovery, and (2) low overlap with previous results (DeSalvo et al., 2008). In our previous experiment greater than 20 % of genes were found differentially expressed upon induction of bleaching (DeSalvo et al., 2008). These discrepancies might be explained by differences in experimental procedures such as: acclimation time (4 days vs. 21 days), initial rate of temperature change ($0.73\text{ }^{\circ}\text{C h}^{-1}$ vs. $0.35\text{ }^{\circ}\text{C h}^{-1}$), sampling time points, water quality, light regime, etc. However, we provide strong evidence that the symbiont genotype had a substantial influence on host gene expression (see below), and thus diminished our ability to detect more genes differentially expressed during thermal stress and recovery. Nevertheless, we found an enrichment of genes related to transcription regulation and stress response (Figure 4.2). Interestingly, a number of those genes are dif-

ferentially expressed between control and recovered samples. These genes (e.g., heat shock factor protein) represent candidates for future studies given their possible involvement in the acquisition of experience-mediated thermal stress resistance (Brown et al., 2002).

The results from comparing control and stressed fragments from the same host genotype, an approach that allowed us to disentangle the contribution of host genetic background and the *Symbiodinium* type hosted, yielded a low number (about 2 % of all assayed genes) of differentially expressed genes with fold-changes less than 1.53. This is in stark contrast to the single-genotype experiment described in DeSalvo et al. 2008, where 24 % of all assayed genes were found differentially expressed. Intriguingly, all fragments in the 2008 study hosted the same *Symbiodinium* genotype (clade A), which, besides methodological differences, supports the notion that the host gene expression is correlated with the symbiont-genotype hosted.

Based on the results found in this study, i.e. an influence of *Symbiodinium* genotypes on coral transcriptomes, and the variability in transcriptomic responses seen in different experiments, the identification of a core set of stress-responsive genes represents a daunting challenge. Such an endeavor will require accounting for differences in both abiotic and biotic factors such as experimental procedures (acclimation conditions, rate and magnitude of temperature increase, stress exposure time, etc.) and host and symbiont genotypic backgrounds, respectively.

Integration of Host Gene Expression and Symbiodinium Genotyping Analyses

Numerous studies support the notion that different symbionts have far-reaching effects on host physiology. Growth rates in *Aiptasia pulchella* (Kinzie and Chee, 1979) and in juvenile *Acropora millepora* and *Acropora tenuis* (Little et al., 2004; Mieog et al., 2009) are dependent on the symbiont genotype. Host growth is likely dependent on nutrient availability; thus, differential growth as a result of symbiont genotype may be related to the type (Loram et al., 2007) and amount (Cantin et al., 2009; Stat et al., 2008) of nutrients translocated from the symbiont to the host. Furthermore, a recent study investigated growth, survival, and thermal tolerance in different host-*Symbiodinium* combinations and found that *Symbiodinium* type was a better predictor of holobiont fitness than host genetic effects (Mieog et al., 2009). Finally, during natural bleaching of *Montastraea* spp., clade A- and C-associated colony regions were less susceptible to thermal stress compared to clade B- and C-associated regions (Rowan et al., 1997).

In both of our experiments, acclimation of field-collected samples to the same light and temperature conditions for 4 weeks did not cause homogenization of *Symbiodinium* genotypes as seen in the control samples. Thus, it is possible that the observed patterns in host gene expression could have resulted from long-term acclimatization effects (months, years) to different environmental conditions in the field (e.g., light levels and temperature). The homogenization of *Symbiodinium* genotypes following thermal stress (all recovered fragments hosted *Symbiodinium*

clade A) could also be interpreted as host physiology determining symbiont type dominance or alternatively, symbiont type content driving host gene expression. Taken together, we acknowledge that our experimental design did not allow disentangling a cause and effect relationship, i.e. whether a change in environmental conditions changed host physiology, and in turn a change in *Symbiodinium* type dominance, or that a change in environmental conditions caused a change in *Symbiodinium* type dominance and in turn a change host physiology (reflected in transcriptomic states). Nevertheless, the congruency between the array tree groupings and the *Symbiodinium* community compositions (Figure 4.3), which is seen in both experiments presented here, constitutes strong evidence that there exists a correlation between different *Symbiodinium* genotypes and the state of the coral host transcriptome. Our statistical analyses corroborate these findings in that the greatest differences in gene expression were found between clade B hosting top fragments and clade C hosting middle/bottom fragments (Figure 4.4). However, in thermally stressed top, middle, and bottom samples the correlation notably diminished as seen by the lower number of differentially expressed genes compared to the corresponding analysis in control samples (Figure 4.4). The observed reduction was due to genes becoming more similar in their expression during stress rather than an increase in variation (Table S3).

The comparison between top, middle, and bottom fragments suggests that pathways involved in protein translation, folding, and degradation, in addition to other genes listed in Figure 4.5, are differentially affected in coral fragments hosting different *Symbiodinium* genotypes. Since these results are based on a single host genotype and a relatively small microarray, they set the stage for an in-depth analysis of differentially regulated pathways involving a multi-colony experimental design. If we are to succeed in such an effort, then surveying host and symbiont genotypic diversity must precede and thus inform the design of future experiments. At the time of writing, a *M. faveolata* microarray containing greater than 10000 features is under fabrication, which will be utilized in the future to address this important gap of knowledge.

Conclusions and outlook

While the order of events, i.e. host physiology driving symbiont content and/or symbiont content driving host physiology, requires further investigation, the convergent findings of the two experiments outlined in this study strongly argues for the existence of a correlation between host transcriptional states and the symbiont genotype. It would thus be interesting to hypothesize that different symbiont types may modulate the host transcriptome in more or less stress-responsive configurations, which would add a host transcriptomic perspective to a *Symbiodinium*-centric view of bleaching susceptibility.

Advancement in the understanding of the apparent differences observed in bleaching susceptibility of different coral-*Symbiodinium* partnerships are of particular relevance given the grim outlook of coral populations in the face of large-scale

climate change-induced bleaching events. The ability of some corals to multi-associate most likely arose because symbiont effectiveness changes with environmental conditions (Douglas, 1998). This scenario is consistent with the idea that organisms gain robustness to environmental perturbation by extending their system boundary via the integration of foreign biological entities such as symbionts (Kitano and Oda, 2006). This is exemplified by different coral-*Symbiodinium* genotypic combinations being locally adapted to irradiance (Iglesias-Prieto et al., 2004; Rowan and Knowlton, 1995). So far, only a handful of studies have addressed the effects of mutualistic symbionts on animal host transcriptomes, e.g. human gut microbiome (Hooper et al., 2001), *Vibrio*-squid symbiosis (Chun et al., 2008), and *Symbiodinium*-coral larvae and *Symbiodinium*-anemone symbioses (Rodriguez-Lanetty et al., 2006; Voolstra et al., 2009). These examples illustrate effects on host gene expression upon infection by different symbionts, whereas our study extends this body of knowledge by describing a correlation between symbiont genotype and the host transcriptome in both established symbioses and responses to thermal stress.

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4.8 Supporting information

Supporting information is available online at:

<http://www3.interscience.wiley.com/journal/123276178/supinfo>

4.9 Chapter acknowledgement

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5 General Discussion

It is now generally accepted that most, if not all, animals and plants are associated with microorganisms, presumably ever since the origin of eukaryotes (Margulis, 1970; Hickman, 2005). For example, the total number of cells in the body of a human a superorganism (i.e., including all indigenous microbial cells) is estimated to be 10^{14} , of which only about 10 % are of human origin (Savage, 1977). A microbial gene pool 150 times larger than the human gene complement illustrates the importance that microorganisms may play in the physiology, ecology, and evolution of humans, and by extension all organisms that host microbial associates. Corals are no exception; thus, evaluating and predicting the sustainability of coral reefs will largely depend on our ability to understand the consequences of changing environmental conditions on corals and all its associated microbiota as one functional entity. Capturing the diversity of coral microbiota and elucidating the roles they play within coral holobionts will greatly benefit from a holistic approach driven by the application of post-genomics tools (Figure 5.1).

This thesis represents an initial effort towards such a coral "systems biology" approach (Figure 5.1) that in concert with methods commonly employed in biomedical and veterinary science is anticipated to advance our understanding of the complex interactions between the environment (including disease-causing agents), the coral animal, and the consortium of its associated microbes (Bourne et al., 2009; Work et al., 2008).

5.1 Corals as threatened bacterial habitats

In earlier studies, the prediction of extremely high diversities of coral-associated bacteria and archaea (Rohwer et al., 2002; Kellogg, 2004; Wegley et al., 2004) had already implied that more powerful technologies would be needed to comprehensively capture the microbial diversity. As expected, the massively parallel pyrotag-sequencing approach described in Chapter 2 yielded a much higher resolution of the bacterial community composition when compared to previous studies. This level of resolution made it possible to establish an important link between coral-associated bacteria and members of the "rare" seawater biosphere, i.e., bacterial taxa that are detected in only very low numbers per volume of seawater. Building upon evidence from previous work (Apprill et al., 2009) it is now possible

to hypothesize that (at least some) corals may acquire the complex microbiota present in adult life stages solely by environmental (horizontal) transmission, i.e., without the need to postulate a vertical inheritance of symbionts. Finally, this study represents the most comprehensive microbial census in corals so far, and yielded quantitative insights into the extent of uniqueness of each coral species as a microbial habitat. The implication of these data are that coral holobionts provide specialized habitats for microbiota that will likely be lost, if we fail to conserve coral ecosystems.

The possibility to apply pyrotag-sequencing methods to coral samples represents a major step towards obtaining a comprehensive "parts list" of coral bacterial communities. However, there still remains a gap between the detected and estimated richness of bacterial taxa. Thus, the sequencing effort per sample needs to be increased, if the goal is to completely resolve the bacterial community composition in corals. At the same time, it has been shown that pyrosequencing data tend to be noisy, i.e., they are associated with error rates that inflate the detected and predicted species richness by generating false positives. These errors, however, can now be addressed by improved algorithms (Quince et al., 2009), so that in the future, more empirical data, improvements in sequencing accuracy and throughput, and advancements in data processing methods will likely improve these technical shortcomings.

Important questions that future experiments will need to address (some of which are already in progress) include:

1. What is the intra-individual (within one colony), inter-individual (between colonies of the same species), and interspecific (between different species) variability of bacterial communities in corals?
2. What is the temporal variability of bacterial communities in coral individuals and populations?
3. Which are the key ecological factors that drive regional differences in bacterial community composition?
4. When (and how) are host-bacterial associations established throughout the different life stages of corals?
5. Can patterns of positive (mutualism, cooperation), neutral, or negative (competition) interactions among bacterial taxa be detected once more phylotyping data become available?
6. How many (and which) bacterial taxa are found in all individuals of a coral species, and how many (and which) bacterial taxa are more loosely associated?
7. Are disease-causing agents already present in healthy corals, e.g., as commensals or dormant opportunists?

While these examples are phrased to reflect the bacterial component of the coral holobiont, it is important to note that similar techniques and questions are equally valid and important for other holobiont members.

5.2 Bacterial community shifts in diseased *Montastraea faveolata*

Given the high diversity of bacterial taxa harbored by corals, a hybridization-based technology would represent an attractive alternative to sequencing-based methods. The combination of applying the second generation (G2) of the PhyloChip and sequencing of 16S rRNA gene clone libraries using the same set of samples allowed for evaluating the applicability of this technology to profile coral microbial communities. The results from this study along with those described in the previous section ("Corals as threatened bacterial habitats") concurred in the identification of many previously unknown bacterial taxa. For example, more than 90 % of the 16S rRNA gene sequences could not be classified at the 97 % (OTU) sequence similarity level, while 70 % and 80 % could be classified at the 92 % ("family") and 90 % ("order") sequence similarity level, respectively. As a consequence, the phylogenetic resolution provided by this technology represented a major limitation, which, however, could be compensated by the availability of nearly full-length 16S rRNA gene sequences. In fact, the combined analysis of these two types of data suggested that bacteria previously identified in other stressed, diseased, or injured marine invertebrates were differentially more abundant in diseased coral samples, which provides evidence for the validity of the results.

Despite the shortcomings mentioned above, some bacterial species that are thought to associate with corals were very well represented on the PhyloChip(G2). The absence of evidence for the presence of the putative pathogen *Aurantimonas coralllicida* in diseased samples lead to the proposition that similar symptoms seen on different coral species may involve different etiologies, and that previous generalizations assuming a broad host-range of this pathogen need to be re-evaluated.

Increasing the phylogenetic resolution of the PhyloChip represents an important goal to foster its application for bacterial community profiling in corals. The addition of about 2,500 nearly full-length 16S rRNA sequences derived from coral samples to the sequence pool that was used to design the next PhyloChip generation (G3) has been an important step in this direction. In fact, preliminary data resulting from the application of the current PhyloChip(G3) show that newly formed coral-associated OTUs are detected with high fidelity in a set of healthy and diseased coral samples (C. Closek, pers. comm.). This follow-up experiment examines whether healthy-looking parts of a diseased colony already displays disease-like signatures in the bacterial community profile.

So far, the samples used for PhyloChip profiling of bacterial communities in corals represent static "snap shots" of diseased corals. Future studies will need to address the temporal resolution of disease progression as well as incorporate methods commonly employed in biomedical and veterinary science (e.g., histological description of lesions, application of Kochs postulates) to advance our understanding of coral diseases (Work et al., 2008). From a systems biology point of view, the

interactions between disease-causing agents, the holobiont, and the environment will need to be integrated. Thus, simultaneous monitoring of abiotic factors (temperature, nutrients, etc.) and profiling the microbial community composition of the waters surrounding corals before, during, and after disease episodes are envisioned for future studies.

5.3 Coral transcriptome linked to *Symbiodinium* genotypes

With the accomplishment of various cDNA sequencing projects (e.g., Schwarz et al., 2008), gene-expression microarrays have recently become available to conduct mid- to large-scale transcriptomic studies in corals. In the last 4-5 years, a multitude of publications reported the usage of cDNA microarrays predominantly to examine environmental stress responses (e.g., DeSalvo et al., 2008; Grasso et al., 2008; Richier et al., 2008; Voolstra et al., 2009), or gene expression differences in different developmental stages of corals (Grasso et al., 2008; Reyes-Bermudez et al., 2009). However, the study described in Chapter 4 represents the first study of its kind to link different symbiont genotypes to transcriptomic states in corals. These results highlight the importance of integrating the roles that microorganisms have played in the physiology, ecology, and the evolution of the coral holobiont (Zilber-Rosenberg and Rosenberg, 2008).

5.4 Conclusions and perspectives

In a traditional sense, systems biology emerged as the result of the "genomic revolution" since gene catalogs of entire organisms have become available. Simultaneous advances in high-throughput technologies and computational power have propelled a shift in biological research from reductionistic to system-level approaches. The Institute for Systems Biology (Seattle, WA) dubbed "systems biology" as the 21st century science and defined it as "the study of an organism, viewed as an integrated and interacting network of genes, proteins and biochemical reactions which give rise to life". In light of the importance microorganisms play in the functioning of a host, this definition could be extended to reflect both the holobiont as a functional unit and its interplay with the environment. Systems biology is a nascent field with a bright future; at the same time, coral reef ecosystems, at least as we know them today, are facing extinction. Thus, rapid advances in understanding coral health and disease are imperative to predict the future of coral reefs and guide the implementation of effective management strategies.

The previous chapters are case-examples of: 1) profiling the host transcriptome (Chapter 4), 2) phylotyping (Chapter 2) and comparing (Chapter 3) the bacterial community composition, 3) genotyping coral hosts and *Symbiodinium*

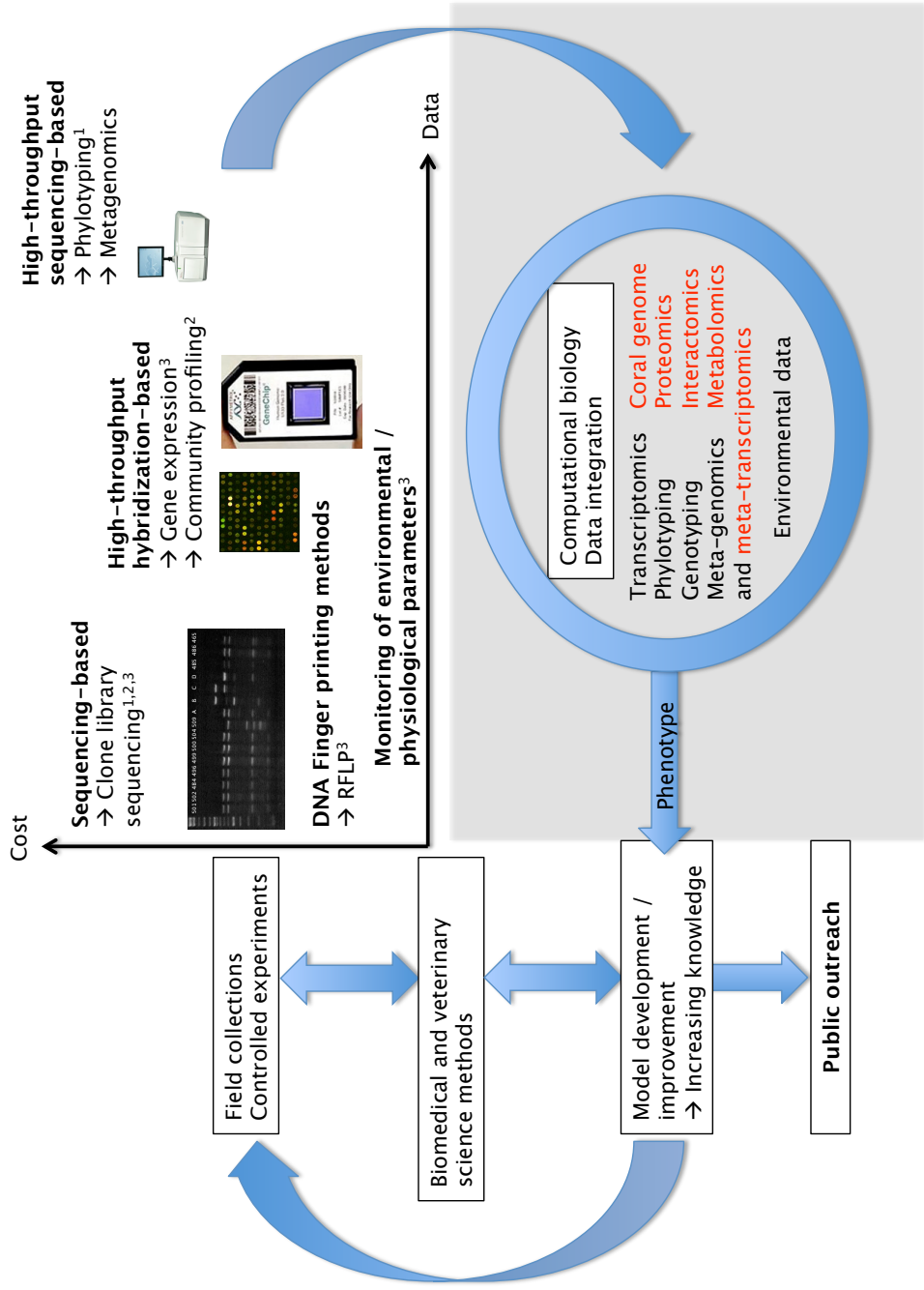


Figure 5.1: Status and future of coral systems biology. Methods that are available to assay the coral transcriptome, microbial community composition, and the microbial gene pool are shown as a cost-data function (top, right). Computational methods will drive the integration of data that are (or in the near future will become) available (bottom, right) to improve existing models and our understanding of coral holobiont health and disease in a changing environment. The resulting models can be tested by new hypothesis-driven experiments and scrutinized by established biomedical approaches (left).

populations (Chapter 4), and 4) monitoring physiological and environmental data (Chapter 4) using naturally (Chapter 3) or experimentally disturbed (Chapter 4) coral holobionts. The integration of these data types with the imminent availability of the first coral genome and other high-throughput technology data (e.g., metagenomics, metatranscriptomics, proteomics, and metabolomics) are expected to give rise to a field that could be defined as "coral (eco)systems biology" (Figure 5.1). A main focus will need to be placed on the incorporation of the role of microorganisms that have the potential to respond to environmental change within ecological time frames. The question whether and how microbial communities relate to macro-ecological (Ainsworth et al., 2009) or evolutionary changes (Zilber-Rosenberg and Rosenberg, 2008) could be addressed by metagenomics or comparative genomics approaches. Ultimately, a broadly scaled systems approach could improve current models of coral holobiont health and disease in a changing environment; and as such, it keeps the promise to predict, prevent, or remedy the decline of coral reefs: the yet most diverse marine ecosystems in the world.

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