The dynamics of bacterial populations associated with corals and the possible role of bacterial pathogens in coral bleaching

Thesis submitted by

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STATEMENT ON SOURCES

Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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(Signature) (Date)

STATEMENT ON THE CONTRIBUTION OF OTHERS

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ABSTRACT

The coral holobiont is known to comprise a diverse array of microbial partners, including the dinoflagellate endosymbiont, *Symbiodinium,* and bacteria living both on and within coral tissues, but little is known about the contributions that bacterial communities make to the overall partnership or how they interact with other microbial partners. Research described in this thesis aimed to understand coral- associated bacterial communities on the Great Barrier Reef and the nature of their interactions with *Symbiodinium* partners.

Bacterial diversities documented on three common reef corals, *Acropora millepora*, *A. tenuis* and *A. valida*, confirmed that corals associate with specific microbiota. According to three culture-independent techniques [denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (t-RFLP) and clone libraries], consistent bacterial profiles were also conserved among all three species of *Acropora* within each of two study locations (Magnetic Is and Orpheus Is reefs), suggesting that closely related corals of the same genus harbor similar bacterial types. Bacterial community profiles of *A. millepora* at Orpheus Island were consistent throughout the year, indicating a stable community despite seasonal variation in environmental parameters. However, clone libraries, DGGE and t-RFLP profiles revealed bacterial communities grouped according to location rather than coral species.

To further investigate the influence of environmental factors on coral-microbial associations, adult colonies of *A. millepora* were reciprocally translocated between two nearshore reefs (Magnetic Island and Great Keppel Island reefs) that differed in temperature. Clone libraries and DGGE profiles for corals placed on

galvanized iron racks revealed that bacterial communities differed from those associated with *in situ* corals at Magnetic Island. Lack of change in bacterial communities of translocated corals through two bleaching events (a cold water bleaching on Great Keppel Island reef and a warm water bleaching at Magnetic Island reef) suggests that the rack environment of translocated corals was more important in shaping bacterial communities associated with *Acropora millepora* than the reef environment to which they were translocated. Nutrient inputs, such as metal derived Fe^{2+}, may have influenced the bacteria present on translocated corals and should be considered in manipulative studies.

A study comparing bacterial communities on 9-month old juvenile corals hosting either type C1 or D *Symbiodinium* suggested that coral-associated bacteria are not linked to *Symbiodinium* type *in hospite,* at least during early ontogeny. However, in contrast to bacterial profiles of adult corals, bacterial communities associated with juvenile corals were highly variable, indicating that bacterial associates are not conserved in these early stages. When 12-month old juveniles were sampled again in summer, bacterial communities associated with *A. tenuis* hosting clade D *Symbiodinium* were dominated by sequences affiliating with *Vibrio* species, indicating that corals harbouring this symbiont may be more susceptible to temperature stress, allowing growth of opportunistic microbial community members, possibly detrimental to coral health.

To further investigate the role that temperature may play in the complex interactions of the coral holobiont, a controlled temperature experiment was undertaken with bacterial community shifts assessed in relation to coral *Symbiodinium* type. Shifts in bacterial associates on juvenile corals harboring ITS 1 type D *Symbiodinium* were observed when placed in a high (32°C) temperature treatment. In

particular, there was a marked increase in the number of retrieved *Vibrio*-affiliated sequences, which coincided with a marked decline in their photochemical efficiency. In contrast, *A. tenuis* hosting ITS 1 type C1 *Symbiodinium* did not exhibit major bacterial shifts in the elevated temperature treatment, indicating a more stable bacterial community during thermal stress; concomitantly a decline in photochemical efficiency was minimal for this group. The lower resilience of *A. tenuis* to thermal stress when harbouring *Symbiodinium* D highlights the importance of inter-kingdom interactions among the coral host, dinoflagellate endosymbiont and bacterial associates for coral health and resilience.

Comparisons of healthy vs. bleached coral metagenomic datasets revealed major shifts in microbial associates during heat stress, including shifts in *Bacteria*, *Archaea*, viruses, *Fungi* and micro-algae. The microbial community shifted from an autotrophic to a heterotrophically-driven metabolism, as evidenced by increases in fatty acid, protein, simple carbohydrate, phosphorus, and sulfur metabolism genes. The proportion of virulence genes was also higher in the bleached sample, indicating that bleaching may contribute to an increase in microorganisms capable of pathogenesis. These results demonstrate that thermal stress can result in shifts in coral-associated microbial communities, which may lead to deteriorating coral health.

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Chapter 1.0 Background and General Introduction

1.1 Coral reefs under threat

Coral reefs are among the most biologically diverse ecosystems on the planet, and vital to the sustainability and prosperity of communities that reside in coastal regions around these reefs (Moberg ant Folke 1999). Through fisheries, coastal protection, building materials, biochemical compounds and tourism, it is estimated that the economical value of coral reefs per year is US $375 billion (Costanza et al. 1997). However, reefs are under pressure and in some places in rapid decline. Currently, it has been estimated that ~19% of coral reefs have been effectively destroyed through direct human pressures with no immediate prospects of recovery (Wilkinson, 2008). An additional 15% are under imminent (10-20 years) risk of collapse, and another 20% are under a longer-term (> 20 years) threat (Wilkinson, 2008). Given the fundamental socio-economic roles coral reefs have in many countries, it is vital that these ecosystems are better understood and protected.

Pollution due to run-off from agriculture and land-development, as well as overfishing, have contributed to localized destruction of reefs (Hughes 1994, Jackson et al. 2001, Pandolfi et al. 2003,). Compounding these threats are the effects of climate change caused by rising anthropogenic CO_2, which has resulted in an estimated warming of the world's average ocean temperatures by 0.74°C during the 20th century (IPCC 2007). As a result of escalating seawater temperatures, massive bleaching has become one of the foremost threats to the world's coral reefs (Goreau et al 1997, Hayes and Goreau 1998, Hoegh-Guldberg 1999, Hughes et al. 2003). Coral bleaching involves the breakdown of the symbiotic relationship between *Symbiodinium* (zooxanthellae) and the coral host that can potentially lead to coral mortality. Since the 1980s, the severity and frequency of mass coral bleaching has increased (Glynn

1993, Brown 1997, Hoegh-Guldberg 1999). However, the dramatic rise in atmospheric carbon dioxide has been mitigated by the ocean carbon sink, with approximately one-third of the CO_2 emitted in the last 20 years sequestered by the ocean (Sabine et al. 2004). As a consequence, surface seawater pH has decreased due to the acidifying effects of CO_2 in water (Caldeira and Wickett, 2003), in turn potentially reducing calcification rates of reef-building corals (Kleypas et al. 2006). Furthermore, increased seawater temperatures have been directly linked to increased coral disease abundance and progression (Cervino et al. 2004, Boyett et al. 2007, Bruno et al. 2007). Disease-like syndromes in reef-building corals have become more prevalent over the past two decades (Ward and Lafferty 2004, Harvell et al. 2007) with new diseases emerging and observed in areas not previously found (e.g. Aeby 2005, Dalton and Smith 2006, Haapkyla et al. 2007, Korrubel and Riegl 1998). The recent trends in coral health suggest that microbial associations have become destabilized as a result of environmental disturbance (Knowlton and Rohwer 2003). Therefore, it is important to understand the symbiotic relationships of corals and their microbial associates and how these relationships can break down due to stress.

1.2 The coral holobiont

Reef-building corals harbour a highly diverse community of microbes, including algae, *Bacteria*, *Fungi*, *Archaea* and viruses. Photosynthetic dinoflagellates in the genus *Symbiodinium* (zooxanthellae) are considered to be a keystone symbiont of corals, providing a source of carbon necessary for coral growth (Muller Parker and D'Elia 1997). Less known are the roles of the other associates that populate the mucus and tissue layers of corals. Microbes are responsible for biogeochemical cycling within pelagic, oligotrophic waters through a series of processes that together comprise the microbial loop (Azam et al. 1983). The diverse metabolic capabilities of bacteria

led to the hypothesis that microbes associated with corals may break down the byproducts of *Symbiodinium* photosynthesis and coral waste, forming useable nutrient compounds for the coral in a scaled-down version of the microbial loop within the coral host (Rohwer et al. 2002). Furthermore, prokaryotes are effective at scavenging limiting nutrients necessary for primary productivity (Thingstad et al. 1998, Behrenfeld and Kolber 1999, Cavender-Bares et al. 2001) and are a source of antimicrobial compounds (Castillo et al. 2001, Ritchie 2006). Microbes may, in return, benefit from the coral as a source of nutrients (Rublee et al. 1980, Herndl and Velmirov 1986) or UV radiation protection provided by the coral mucus. Rohwer et al. (2002) thus introduced the concept of the coral as a holobiont, comprised of a complex consortium consisting of the coral host and its many symbiotic microorganisms that inhabit the mucus, tissue layers and skeleton and perform necessary functions for the coral organism (Figure 1). Therefore, knowing what organisms inhabit corals and what each contributes to the partnership will aid our understanding of this multi-species mutualism and identify key players necessary to maintain the health of reef-building corals. The genetic diversity of microbial associates has begun to be elucidated using the tools of molecular biology. However, the utility of many microbial partners remain speculative.

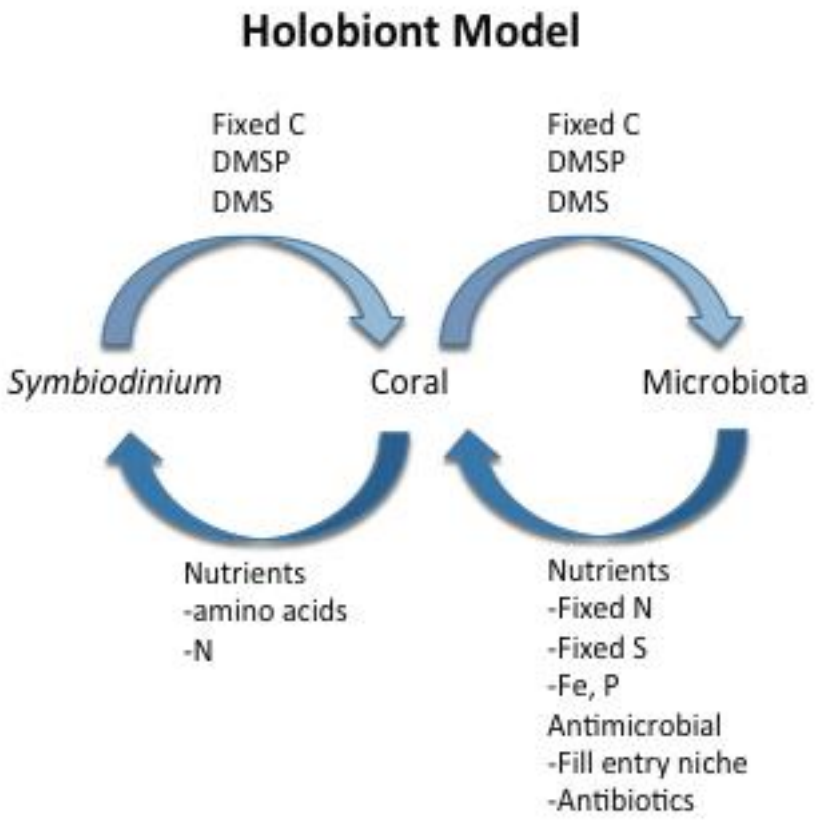

Figure 2.1 Proposed model of a coral colony as a holobiont. Arrows indicate contributions by holobiont members. Figure adapted from Rohwer et al. (2002).

1.2.1 Symbiodinium *diversity and function*

Symbiodinium are well known for their symbiotic partnerships with corals, providing photosynthetic carbon and inorganic nutrients essential for their hosts (reviewed in Muller-Parker and D'Elia 1997). This relationship is obligatory for zooxanthellate corals, consequently the sustenance endosymbionts provide is critical to the productivity of reef systems (Stanley and Swart 1995).

Zooxanthellae were initially identified as one species, *Symbiodinium microadriaticum* (Fruedenthal 1962), but they are now recognised to be a diverse collection of organisms with members belonging to the classes *Bacillariophyceae*, *Dinophyceae*, *Rhodophyceae*, and *Cryptophyceae* (reviewed in Blank and Trench 1986). Most zooxanthellae belong to one of the eight genera of dinoflagellates, and

have been identified as endosymbionts of various marine invertebrates and protists (Banaszak et al. 1993, Trench 1997). The most studied genus of symbiotic dinoflagellates that predominate within reef systems, is *Symbiodinium*. There are currently eleven named species of *Symbiodinium*, as well as several lineages, designated as clades A through H, that have been established based on phylogenetic analysis of the nuclear ribosomal DNA operon, including the nuclear ribosomal small subunit 18S (Rowan and Powers 1991, McNally et al. 1994), large subunit 28S (Wilcox 1998, Baker 1999, Pochon et al. 2001), and internal transcribed spacer (Hunter et al. 1997, LaJeunesse 2001). The phylogenetic resolution of *Symbiodinium* taxonomic classifications, however, remains ill-defined. Genetic indicators such as isozymes, randomly amplified polymorphic DNA, DNA fingerprinting and microsatellites have revealed hundreds of possible distinct genotypes (Baille et al. 1998, 2000, Goulet and Coffroth 1997, 2003; Howells et al. 2009). The genus appears to be speciose (Blank and Trench 1985), but currently *Symbiodinium* are classified according to clades or subgenera, each likely consisting of multiple species (Baker 2003). Furthermore, only a small number of hosts (scleractinian corals, soritid foraminifera, gorgonians and tridacnid clams) have been used for culture of *Symbiodinium* and genetic analysis (see Baker 2003 for review, Baker 2001, Rowan and Powers 1991, Santos et al. 2002), while only a few studies have identified *Symbiodinium* free-living in the ambient environment (Loeblich and Sherley 1979, Carlos et al. 1999, Gou et al. 2003, Coffroth et al. 2006, Littman et al. 2008). Therefore, *Symbiodinium* genetic diversity remains largely unexplored. Nevertheless, the high level of diversity in symbionts suggests that hosts have a wide variety of possible dinoflagellate partners available to them.

1.2.2 Bacterial diversity and function

Prokaryotes associated with corals are both diverse and abundant (DiSalvo and Gundersen 1971, Sorokin 1973, Mitchell and Chet 1975, Ducklow and Mitchell 1979, Pascal and Vacelet 1981, Segel and Ducklow 1982, Herndl and Velimirov 1986, Paul et al. 1986, Williams et al. 1987, Shashar et al. 1994, Ritchie and Smith 1995, 1997, Santavy 1995, Santavy et al. 1995, Kushmaro et al. 1996, Santavy and Peters 1997, Rohwer et al. 2001, Frias-Lopez et al. 2002, Rohwer et al. 2002). Culture-independent techniques have enabled investigators to identify a wide diversity of microbial groups by sequencing the 16S rRNA gene. Rohwer et al. 2002 examined three Caribbean coral species and identified 430 distinct bacterial ribotypes originating from 14 coral samples. Statistical analysis of these diversity studies inferred approximately 6000 bacterial ribotypes associated with these coral species. Half of the sequences retrieved shared less than 93% identity with previously identified 16S rRNA gene sequences, indicating a high number of novel species and genera associated with these corals (Rohwer et al. 2002). Similarly, Sunagawa et al. (2010) used pyrosequencing and found 8,500 different operational taxonomic units among seven different coral species, and those that dominated on corals were found to be rare bacterial species. Additional studies examining other species of corals from different regions have noted similar levels of diversity richness (Rohwer et al. 2001, Friaz-Lopez et al 2002, Bourne and Munn 2005, Lampert et al. 2008, Hong et al. 2009, Kvennefors et al. 2010) demonstrating the profusion of coral-microbial associations that occur and the need for intensive sequencing efforts to reveal the full diversity of these associations.

1.2.2.1 Bacterial specificity

The bacterial communities of corals differ from those of the overlaying water column (Rohwer et al. 2001, Frias-Lopez et al. 2002, Kvennefors et al. 2010) implying that the bacteria residing on corals establish specific associations. Several studies have noted that bacterial communities from the same coral species were similar despite geographical separation, but that the abundance and types of bacterial groups varied among coral species, suggesting that coral associations with microbiota are potentially species-specific. For example, analysis of the microbial community on the Caribbean coral, *Monastraea franksi* revealed that the bacterial community was similar for 25 samples over 5 different reefs spaced 10km apart (Rohwer et al. 2001). Rohwer et al. (2002) found that the microbial groups differed between *Porite astreoides, Diploria strigosa* and *Montastraea franksi*, but were similar within species, despite the samples originating from different Caribbean sites. Lampert et al. (2008) compared bacterial communities of *Platygyra lamellina* and *Fungia scutaria* and similarly found very different bacterial profiles. Analysis of DGGE profiles for 69 samples from 2 coral species confirmed that bacterial associations were species-specific, but differed significantly between *Acropora hyacinthus* and *Stylophora pistillata* (Kvennefors et al. 2010). Bourne and Munn (2005) examined *Pocillopora damicornis* on the GBR, and similarly found conservation of bacterial groups between coral colonies within a species, but discovered that microbial communities differ between the surface mucus layer and coral tissues, demonstrating that the coral is composed of distinct micro-niches. Interestingly, one γ-proteobacterial sequence obtained from samples from the GBR (Bourne and Munn 2005) was also retrieved in previous studies in the Caribbean (Rohwer et al. 2001, Cooney et al. 2002, Frias-Lopez et al. 2002), indicating that coral-associated bacteria can be globally distributed. The specificity of coral-bacteria associations has led to the idea that microbial communities are maintained to carry out

specific functions within the coral holobiont. However, analysis of *Stylophora pistillata* from Taiwan revealed that corals do not always exhibit conserved bacterial communities (Hong et al. 2009). Replicate coral colonies within and between locations and between different seasons displayed highly variable community structures, indicating that seasonal factors, geographical factors and coral physiology can potentially affect the bacterial associates harboured (Hong et al. 2009). Therefore, the relationship between bacteria and coral host may be more dynamic and complicated than previously believed.

1.2.2.2 Nutrient cycling

The roles of prokaryotic partners in the multipartite symbioses are virtually unknown, although some potential benefits can be interpreted based on the characteristics of the bacteria identified. The functions of coral-associated bacteria can be generally classified as either biogeochemical cycling within the coral or production of protective properties against predation and disease. Some types of bacteria associated with corals have been implicated in cycling nitrogen, sulfur and carbon (Williams et al. 1987; Szmant et al. 1990; Shashar et al. 1994; Lesser et al. 2007; Wegley et al. 2007; Chimetto et al. 2008; Olson et al. 2009; Raina et al. 2009; Kimes et al. 2010). Corals have been observed to consume their own mucus (Coles and Strathmann 1973), suggesting that any contribution by the microbial community to the mucus layer may serve as a food source for corals. Since corals generally inhabit oligotrophic waters that are nutrient deficient, biogeochemical cycling by coral-associated bacteria is likely to be crucial for the functioning of coral reef ecosystems. Due to photosynthate contributions by *Symbiodinium* and its nutrient trapping abilities, coral mucus is an important carrier of energy and nutrients and a valuable substrate for microbial communities in the sediment and water column (Wild et al. 2004). Bacteria

are dependent upon these nutrients, therefore contributions to the mucus by *Symbiodinium* may determine which microbes associate with the coral (Ritchie and Smith 2004; Rosenberg et al. 2007).

For instance, dimethylsulfoniopropionate (DMSP) and dimethyl sulfide (DMS) are produced by certain phytoplankton species (Kirst 1996; Townsend and Keller 1996; Stefels 2000) and high concentrations of DMSP and DMS have been found in corals that host *Symbiodinium* (Van Alstyne et al. 2006; Broadbent and Jones 2006; 2004), implicating *Symbiodinium* as a potential source of sulfur compounds on coral reefs. Raina et al. (2009) reported several coral-microbial associates capable of degrading DMS, DMSP and acrylic acid, and these associates comprised a large portion of the coral's bacterial clone libraries. Furthermore, Raina et al. (2009) demonstrated the presence of genes involved in DMSP metabolism in some bacterial isolates, confirming the ability of bacteria to break down these sulfur sources. Similarly, Wegley et al. (2007) noted genes present for transport and degradation of gluthionine as a sulfur source in the metagenome of *Porites astreoides*. Therefore, the bacteria harboured by corals have the potential for cycling sulfur on coral reefs.

Coral mucus is predominately composed of a carbohydrate complex (Coffroth 1990). Ikeda and Miyachi (1995) estimated that 98% of the net carbon assimilated by *Symbiodinium* is excreted into the coral mucus. Bacteria from 11 different species of corals utilize different carbon sources (Ritchie and Smith 1997), indicating that the carbon source present on corals is important for determining the bacterial community composition. Analysis of the coral's metagenome revealed a large number of genes involved in the uptake and processing of sugars (Wegley et al. 2007). The metabolic capabilities of the microbial community correlate to the compounds found in the

mucus, indicating that the community has evolved to efficiently utilize coral mucus (Wegley et al. 2007).

Symbiodinium growth and abundance are limited by nitrogen availability, and therefore nitrogen is an essential element for maintenance of the symbiotic association (Falkowski et al. 1993, Dubinsky and Jokiel 1994). While corals are able to obtain nitrogen directly from the water column (Muscatine and Delia 1978, Piniak et al 2003), corals may rely on the abilities of microbial associates to fix nitrogen. Based on the acetylene reduction activity of several species of coral, Shashar et al. (1994) demonstrated that nitrogen fixation takes place within the coral holobiont. In addition, cyanobacteria were found to be prevalent within the epithelium of *Montastraea cavernosa* and the presence of nitrogenase indicated that these cyanobacteria were actively fixing nitrogen (Lesser et al. 2004). Nitrogen fixing cyanobacteria have similarly been identified on many other corals (Williams et al. 1987; Shashar et al. 1994; Kayanne et al. 2005; Wegley et al. 2007). The presence of nif-specific regulatory protein and nitrogenase genes within the metagenome of *P. astreoides* indicates that the microbial community is capable of nitrogen fixation. Furthermore, analysis of the nitrogenase gene (nifH) revealed diverse bacteria capable of fixing nitrogen associated with *Montipora capitata* and *M. flabellate* (Olson et al. 2009). Thus, several studies have revealed the metabolic capabilities of bacteria associated with corals, but the actual contributions of these bacteria to the coral holobiont remain speculative.

1.2.2.3 Antimicrobial activity

Bacteria may be responsible for producing chemicals that prevent predation of corals. The source of allelochemicals in corals is unknown, but some may be traced to

bacterial symbionts. Some mutualistic bacteria have been shown to produce secondary metabolites previously believed to be synthesized by corals (Yasumoto et al. 1986, Elyakov et al. 1991). Moreover, it has been proposed that bacteria are necessary for disease resistance (Ritchie and Smith 2004; Rohwer and Kelley 2004; Reshef et al. 2006), through their production of antibiotics and because they out-compete potential pathogens within the surface mucus layer. Antimicrobial properties of tissue extracts have been found for gorgonians and soft corals (Burkholder and Burkholder 1958, Kim 1994, Slattery et al. 1995, 1997, Kelman et al 1998, 2006), as well as for scleractinian corals, including *Pocillopora damicornis* from the Red Sea (Geffen and Rosenberg 2005), *Acropora palmata* from the Caribbean and numerous other Pacific species (Koh 1997). Castillo et al. (2001) examined antimicrobial capabilities of bacterial isolates from various marine organisms and found that 30% of bacteria taken from coral species produced antibiotic substances. Similarly, Ritchie (2006) found that 155 isolates of the 776 culturable bacteria strains investigated inhibited growth of at least one of the bacteria strains tested, and that the antimicrobial properties of the mucus selects for a specific set of commensal bacteria. Rypien et al. (2009) demonstrated that inhibitory activity is present in a majority of co-occurring bacterial associates. Therefore, many bacterial inhabitants have the potential to produce substances that are important for protecting corals and shaping the microbial community structure.

1.2.3 Other associates

The endolithic community resides within the skeleton of corals and consists of cyanobacteria, algae and fungi. Endolithic cyanobacteria have been found to provide organic compounds to *Oculina patagonica* tissue (Fine and Loya 2002) and have been estimated to supply 50% of the nitrogen needs of corals (Ferrer and Szmat, 1988). However, less is known about the diversity and function of endolithic fungi and algae

(Lukas, 1973; Highsmith, 1981; Shashar et al., 1997). The endolithic alga, *Ostroebium quekettii* is common within the skeleton (Lukas 1973). It has been hypothesized that the coral skeleton provides a refuge from UV radiation and herbivory (Shashar et al. 1997) and that the algae, in turn, can serve as an alternative source of photoassimilates for the coral (Fine and Loya 2002). Endolithic fungi are equally as ubiquitous, and typically intermingle with the algae. Although known to be a component of the microbial community of healthy corals (Kendrick et al. 1982; Le Campion-Alsumard et al. 1995a,b; Bentis et al. 2000), it is possible that coral-associated fungi are parasites of corals and endolithic algae (Kendrick et al. 1982; Priess et al. 2000; Golubic et al. 2005). The fungi grow in parallel with the coral's calcium accretion (Le Campion-Alsumard et al. 1995a) and carbonate is most likely deposited at points of attempted penetration by the endolith (Bentis et al. 2000).

Corals also associate with diverse and abundant communities of *Archaea* (Kellogg, 2004; Wegley et al., 2004; Beman et al., 2007). Both mesophilic *Euryarchaeota* and *Crenarchaeota* have been found in association with corals across different reef locations and coral species (Kellogg et al. 2004, Wegley et al. 2004), but these do not appear to be coral species-specific associations (Kellogg, 2004). Presently, the biological role of *Archaea* remains unknown, however their varied and unique metabolic capabilities may be important for maintaining coral health. For instance, Beman et al. (2007) have demonstrated that certain coral-associated *Archaea* are capable of ammonia oxidation, which is necessary for nitrification, and are widespread among coral species and reef locations.

A wide range of virus-like particles regularly occurs in healthy coral tissues, both in the field (Patten et al. 2008, Wegley et al. 2007) and in the laboratory (Wilson et al. 2005). Thus, it has been suggested that viruses may play a role in the coral's

multipartite symbiosis with microorganisms (van Oppen et al 2009). For instance, cyanophages have been found to be the most dominant virus-like particle on corals, suggesting phages may help regulate microbial populations (Wegley et al. 2007). Conversely, viruses have also been linked to coral disease and bleaching (Wilson et al., 2001; 2005; Barash et al., 2005; Davy et al., 2006). Wilson and colleagues (2001) have demonstrated that heat stress in corals can induce a lytic capability in latent viruses, resulting in cell death and spread of the virus. Furthermore, virus-like particles have been observed surrounding and within corals and *Symbiodinium* during UV and heat stress (Davy et al. 2006, Lohr et al. 2007), confirming the importance of latent viral infections during environmental perturbations.

1.3 Dynamics of multi-species mutualisms

Current studies have revealed not only the vast diversity of microbial partners of corals, but also how environmental changes, such as increased sea temperatures, can influence these compositions. This observed flexibility in coral-microbial associations has led to the proposition that the coral holobiont may be able to adjust to changing environmental conditions by altering its symbiont community. This hypothesis has been suggested separately for both *Symbiodinium* (Adaptive Bleaching Hypothesis; Buddemeier and Fautin, 1993) and bacterial communities (Coral Probiotic Hypothesis; Reshef et al. 2006). However, further evidence may suggest that changes in the microbial community better serve as an indicator of community imbalance and suboptimal coral health, as discussed next.

1.3.1 Flexibility of Symbiodinium symbiosis

The distribution of *Symbiodinium* clades and subclades associated with different host taxa display non-random patterns, and the number of host and symbiont

combinations observed are small in comparison to possible combinations (Rowan 1991, Trench 1988,1992), indicating host specificity for certain symbiont types (sensu Dubos and Kessler 1963). However, with the emergence of molecular techniques, recent evidence suggests many, if not all, host taxa are capable of forming associations with multiple types of symbionts. For example, coral hosts can be experimentally infected with a variety of symbiont clades (Fitt 1984, 1985; Schoenberg and Trench 1980; Little et al. 2004; Abrego et al. 2008), and many studies have documented examples of scleractinian corals that can form associations with more than one symbiont in the natural environment (Rowan and Knowlton 1995; Rowan et al. 1997; Baker et al 1997; Baker 1999, 2001; Toller et al. 2001 a,b; Glynn et al. 2001, LaJeunesse 2001, LaJeunesse et al. 2003, Pawlowski et al. 2001, Pochon et al. 2001, Santos et al. 2001, van Oppen 2001, Little et al. 2004; Abrego et al. 2009). Baker (1999) found that 36% of the 107 scleractinian species surveyed in the Caribbean, Eastern Pacific and GBR hosted multiple taxa of *Symbiodinium*. The high level of *Symbiodinium* diversity but the low level of symbiont/host combinations observed might be due to predominance of free-living *Symbiodinium* that rarely inhabit hosts (Baker 2003). However, the presence of low background levels of symbionts in some corals (Ulstrup and van Oppen 2003, Mieog et al. 2007) indicates that the dominance of certain clades may arise due to competition between symbionts within the host (Goulet and Coffroth 1997, LaJeunesse 2002).

The ability to associate with many symbiont types could arguably provide the host with a variety of partnerships, yielding the potential for phenotypic variability in the coral host (Rowan and Knowlton 1995; Rowan et al. 1997; Baker et al. 1997; Rowan 1998; Baker 1999, 2001, 2003, Kinzie 1999; Douglas 2003, Knowlton and Rohwer 2003). Surveys of *Symbiodinium* distributions have revealed depth-related

patterns in symbiont distribution among a variety of hosts. Initial research on *Montastraea annularis* and *M. faveolata* revealed corals hosted *Symbiodinium* A, B, and D in shallow waters as deep as 6m, while *Symbiodinium* C was found in deeper waters, between 3-14m (Rowan and Knowlton 1995, Rowan et al 1997, Toller et al 2001b). Similar patterns were found in other Caribbean scleractinian hosts (Billinghurst et al. 1997; Baker and Rowan 1997; Baker et al. 1997; Rowan 1998; Baker 1999, 2001; Diekmann et al. 2002), as well as differences in subclades of *Symbiodinium* clade C at different depths in corals on the Great Barrier Reef (Baker 1999; LaJeunesse 2003). Such surveys have indicated that shallow, high light habitats tend to have higher symbiont diversity in comparison to deeper, low light environments (Baker 1999; Lajeunesse 2002), most likely due to higher variability in physical conditions typical of shallow reef environments.

1.3.2 The Adaptive Bleaching Hypothesis

The high genetic variation in *Symbiodinium*, combined with the potential for flexibility in coral-algal symbiosis (Rowan and Powers 1991) and the persistence of reef-building corals despite rapid climate changes over geological time scales (Buddemeier et al. 1997) led Buddemeier and Fautin (1993) to propose the Adaptive Bleaching Hypothesis (ABH), which posits that corals can form new symbiotic consortiums that are better suited for otherwise stressful conditions. The adaptability of corals has become an increasingly important debate in regard to the recovery of corals from bleaching. Numerous studies since have documented shifts to more thermally tolerant clade D symbiont types after bleaching events (Glynn et al. 2001; Toller et al. 2001a; Baker et al. 2004; van Oppen et al. 2005; van Oppen and Berkelmans 2006; Jones et al. 2008), confirming the possibility of symbiont change after temperature stress. However, evidence has only recently emerged on how the physiology of the

coral holobiont differs when associated with the different symbiont types (reviewed in van Oppen et al. 2008). Rowan (2004) found that *Pocillipora verrucosa* had higher photochemical efficiency when hosting clade D than clade C when subjected to temperature stress. In another study, Berkelmans and van Oppen (2006) showed that *Acropora millepora* transplanted from the colder southern region of the GBR to a warmer northern location changed symbiont clade from C2 to D after bleaching. Subsequent controlled experiments showed transplanted corals acquired increased thermal tolerance with the new symbiont type. Taken together, these studies imply that corals may be better suited for warmer environments in association with clade D *Symbiodinium*.

However, this conclusion does not take into account host factors or the trade-offs that may be inherent in associating with another symbiont type. Firstly, clade D typically does not dominate symbionts of coral species and is mostly found in shallow waters (Baker 1999; LaJeunesse 2002) or in extremely deep sites (Toller et al. 2001b). Therefore, *Symbiodinium* D may be an opportunistic symbiont, only able to dominate in corals that have been stressed or bleached (Baker 2003). Secondly, association with clade D does not always yield increased thermal tolerance in coral. Abrego et al. (2009) showed that juvenile *Acropora tenuis* exhibited greater photochemical efficiency in photosystem II, higher ratios of maximum net photosynthesis to respiration and lower metabolic costs when hosting clade C1 verses clade D, which implies that host factors are also involved in determining tolerance to heat. Furthermore, there may be compromises for some corals in associating with certain symbiont types. For instance, juvenile *Acropora tenuis* and A. *millepora* experimentally infected with clades C1 grow 2-3 times faster than when infected with clade D (Little et al. 2004), as a consequence of receiving more photosynthates when

partnered with clade C1 (Cantin et al. 2009). As such, shifting associations may result in suboptimal health states for many corals.

1.3.3 Environmental factors driving shifts in coral bacterial communities

Just as the relationship with *Symbiodinium* has proven to be flexible, bacterial associations with corals have been shown to be dynamic in response to changes in environmental conditions. Koren and Rosenberg (2006) examined the bacteria associated with *Oculina patagonica* in summer and winter to establish changes in bacteria over time on healthy corals. While the dominant group (35% of clones sequenced) was *Vibrio splendidus* in both summer and winter, the other 10 most abundant clusters of bacteria differed completely between seasons. This suggests that temperature may have caused a change in bacterial communities even in seemingly healthy corals. Hong et al. (2009) investigated the 16S rRNA gene of bacteria associated with *Seriatopora pistillata* over two seasons in Taiwan and found spatial and temporal variability in bacterial community composition, indicating that seasonal and geographical factors are involved in coral species specificity. Furthermore, Hong and colleagues proposed that wind and flood events were responsible for coastal sediment inputs that caused differences in soil borne bacterial associates. A similar finding was presented by Guppy and Bythell (2006), where spatial and temporal variability in bacteria of the surface mucus layer were attributed to proximity to coastal pollution in Tobago. Moreover, distinct differences have also been found in bacterial communities with depth and along a gradient of coastal pollution, suggesting that proximity to urban centres and UV radiation can affect the bacteria associated with corals (Klause et al. 2007). Thus, several environmental factors can lead to spatial and temporal dynamics of coral-associated bacteria.

1.3.4 Disease-induced changes in bacteria

Coral-associated bacteria can also be dynamic in response to pathogen infection. Several studies have demonstrated qualitative and quantitative shifts in coral-associated microbial populations when comparing healthy versus diseased corals (Ritchie and Smith 1995; Cooney et al. 2002; Frias-Lopez et al. 2002; Pantos et al. 2003; Bourne 2005; Gil-Agudelo et al. 2006; Pantos and Bythell 2006; Sekar et al. 2006; Barneah et al. 2007; Gil-Agudelo et al. 2007; Voss et al. 2007; Sekar et al. 2008; Sungagawa et al. 2009). Barash et al. (2005) demonstrated that *Favia favus* with white plague-like disease harbored a thousand-fold more culturable bacteria, despite the causative agent *Thallosomonas loyeana* comprising only a small portion of the bacterial community (Thompson et al. 2006). When comparing healthy corals with those affected by white plague type 1, Pantos et al. (2003) found that 16S rRNA gene profiles were significantly different. Interestingly, Pantos et al. (2003) also demonstrated that shifts occurred in the bacterial community composition of the whole coral colony, even when just a small part of the colony showed signs of disease. Other studies have similarly shown total bacterial community changes accompanying pathogenic infection (Breitbart et al. 2005; Pantos and Bythell 2006), suggesting that in at least some cases, the diseases are systemic.

1.3.5 Changes in bacterial communities due to stress

Environmental stress may cause shifts in bacterial communities from those dominated by beneficial partners to ones dominated by harmful bacteria. Using tagged colonies followed through a bleaching event, Bourne et al. (2008) demonstrated that the conserved microbial community found on healthy corals changes as the corals bleach, coinciding with the appearance of *Vibrio* sp.-affiliated sequences. The presence

of *Vibrio* sp. is of particular interest, as the members of this genus are often associated with diseased corals (Rosenberg et al. 2007; Bourne et al. 2009). In another study, the microbial community metagenomes of various stressed *Porites compressa* corals were analyzed after exposure to increased temperature, elevated nutrients, dissolved organic carbon and reduced pH (Vega-Thurber et al. 2009). Examination of the functional genes revealed that virulence, stress resistance, sulfur and nitrogen metabolism, motility and chemotaxis, fatty acid and lipid utilization and secondary metabolism increased in stressed corals, along with a shift in microbial community composition. Moreover, most of the metabolic shifts found on thermally stressed corals were attributed to *Vibrio* sp. sequences. These changes in the microbial community structure and function indicate that the coral microbiota may shift towards pathogenicity when the coral holobiont is stressed.

Such infections may be the result of a decrease in coral resistance to disease agents due to loss of beneficial bacteria. Ritchie (2006) found that *Acropora palmata* mucus lost its antibiotic properties after a bleaching event. Only 2% of the bacterial isolates were able to produce antibiotic substances, compared to 20% of isolates from healthy corals. Moreover, *Vibrio* spp. became dominant during bleaching, including species known as coral pathogens, such as *V. shiloi* (Kushmaro et al. 2001) and *V. coralliilyticus* (Ben-Haim et al. 2003a, b). It is difficult to determine whether *Vibrio* overgrowth was due to lack of protective bacteria in the mucus or whether *Vibrio* spp. merely out-compete beneficial bacteria during high temperatures. Regardless, heat-stressed corals seem to become more susceptible to bacterial proliferation as its microbial community changes.

1.3.6 Bacterial-induced bleaching and the rise of the Probiotic Hypothesis

In some instances, *Vibrio* spp. have been shown to cause bleaching with increased seawater temperatures. *Vibrio coralliilyticus* was found to be the causative agent of bleaching and tissue lysis in *Pocillopora damicornis* in the Indian Ocean and Red Sea at elevated seawater temperatures (Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003b). Another well-known example is *Vibrio shiloi*, shown to be the aetilogical agent causing bleaching of *Oculina patagonica* in the Mediterranean (Kushmaro et al. 1996; Kushmaro et al. 1997). During periods of high seawater temperature, *V. shiloi* is able to express virulence factors that enable adhesion to and infection of the coral, as well as subsequent lysis of *Symbiodinium* and coral tissue (Kushmaro et al. 1997; Banin et al. 2000; Banin et al. 2001; Banin et al. 2003). Despite being an established coral/bacterial infection model system, infection trials became unsuccessful after a number of years, potentially due to acquisition of pathogen resistance by the corals. This led to the Coral Probiotic Hypothesis, which postulates that corals may alter their bacterial community such that the coral holobiont can adapt to changing environmental conditions (Reshef et al. 2006). Given that corals host a highly diverse community of bacteria and that these associations have been found to be dynamic in response to environmental perturbations, Reshef et al. (2006) proposed that corals are capable developing resistance to pathogens. This was further supported by a similar development of resistance following an outbreak of white plague type II, first observed in Florida by Dustan (1977). Although a severe outbreak occurred in 1995 (Richardson et al. 1998), healthy corals are no longer susceptible to the disease (Richardson and Aronson 2002). The fungal disease, Aspergillosis, which infects gorgonians, was first documented in the Caribbean in 1995 (Nagelkerken et al. 1997) and nearly eradicated sea fans at some sites. However, the epizootic has

declined over the years (Kim and Harvell 2004). Since other factors that may contribute to the spread of the disease, such as increased temperature, African dust inputs and exposure to nutrients, have continued, it is likely that there is increased resistance to the disease among sea fans (Kim and Harvell 2004). While corals are not considered to possess an adaptive immune system, recent evidence indicates that corals possibly have another mechanism for acquiring resistance to pathogens. Therefore, future studies should explore whether microbial communities shift to enable corals to adapt to stress.

1.4 Interactions between microbial constituents

Since corals are a multispecies mutualism, it is highly likely that interactions between various microbial partners in the coral holobiont occur. For example, the *Symbiodinium* type affects the physiology of the coral holobiont, as discussed above (section 1.3.2), and therefore direct or indirect effects on other microbial inhabitants are also likely. Two studies have demonstrated differences in disease susceptibility in relation to the *Symbiodinium* type harboured, which suggests that microbial community structure can be affected by the *Symbiodinium* clade *in hospite*. For instance, Sussman et al. (2009) showed differential susceptibility of *Symbiodinium* clades to PSII inactivation by a *Vibrio*-derived metalloprotease. Thus, corals harbouring certain types of *Symbiodinium* could be more resistant to bacterial-induced bleaching. Stat et al. 2008 found differences in health states of *Acropora cytherea* when harboring different clades of *Symbiodinium*. Corals appeared to be more susceptible to disease agents when in association with clade A *Symbiodinium* than clade C. This was attributed to less carbon fixed and passed on to the coral by *Symbiodinium* A, causing a lower resistance of the coral to disease. Further work is

clearly necessary to clarify the interactions between coral associates in order to better understand how shifts in endoymbionts affect the health of the coral holobiont.

1.5 Questions not fully answered

To date, very few studies examining microbial diversity associated with corals have been carried out on the Great Barrier Reef (but see Bourne and Munn 2005; Bourne et al. 2008). It is important to establish a baseline for the diversity of coral-associated bacteria on the GBR in order to identify healthy microbial communities and specific species important in maintaining coral health. It is equally important to determine which factors contribute to the dynamics of the bacterial partnerships, for example the potential for coral-associated microbial communities to vary spatially, temporally, and among coral hosts. Each of these factors may cause natural changes in the microbial associates.

Little is known about the development of coral-bacterial associations. Recent evidence indicates that bacteria are acquired horizontally from the water column during larval development (Apprill et al. 2009), therefore it is possible that the bacterial community structure changes throughout the coral's development. As such, it is important to examine the bacteria at different stages of the coral's ontogeny.

As a multi-species mutualism, it is also important to know if microbial partners interact. This may have implications for the resilience of corals to environmental stress, given that changes in the bacterial community structure may lead to changes in its function. Therefore, it is also important to understand how *Symbiodinium* types affect coral-associated bacteria during times of environmental stress.

Finally, what are the effects of stress on the dynamics of the corals' microbial partners? Based on recent evidence, it appears that heat stress causes a shift in

microbial associates from beneficial to pathogenic partners (Ritchie 2006; Bourne et al. 2008), which may ultimately lead to secondary infections that can be fatal. Therefore it is important to determine functional changes in the coral microbial community during times of stress to confirm whether these shifts are detrimental to the coral.

1.6 Objectives and thesis outline

This research project aims to investigate temporal and spatial dynamics of coral-associated bacterial communities and the effects of environmental change (specifically temperature increases) on these bacterial communities. The specific objectives of this research are to:

1. Determine if bacterial community composition differs spatially and temporally among three related coral species on the Great Barrier Reef.

2. Determine if a change in environment via translocation of corals to another site affects the associated bacterial community.

3. Examine differences in bacterial diversity associated with the corals *Acropora millepora* and *A. tenuis* when hosting two different types of *Symbiodinium* and at different ontogenetic stages.

4. Determine if heat stress affects coral-associated bacterial communities differently on corals hosting different types of *Symbiodinium*.

5. Employ metagenomic techniques to analyse bacterial communities associated with *Acropora millepora* before and during a natural bleaching event in 2002 on the Great Barrier Reef, to gain further insights into metabolic and taxonomic shifts occurring during heat stress *in situ*.

Chapter 2.0 Spatial and temporal variability of coral-associated bacteria on the Great Barrier Reef

This chapter is inserted without abstract as published in the journal *FEMS Microbiology Ecology*:

Littman, R. A., Willis, B. L., Pfeffer, C. and Bourne, D. G. (2009) Diversities of coral-associated bacteria differ with location, but not species, for three acroporid corals on the Great Barrier Reef. *FEMS Microbiology Ecology*. **68**: 152-163.

All the data was collected and analyzed by R. Littman, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.

2.1 Introduction

Bacteria associated with corals are both diverse and abundant (Ritchie & Smith, 1995, 1997; Santavy, 1995; Kushmaro et al., 1996; Shashar et al., 1996; Rohwer et al., 2001, 2002; Frias-Lopez et al., 2002); however, current understanding of how this diversity varies among coral species is limited. Microbial communities occupy a range of niches on corals, from within the surface mucus layer (Ducklow & Mitchell, 1979; Paul et al., 1986; Ritchie & Smith, 1995, 2004; Bourne & Munn 2005) to both on and within the coral tissue layers (Williams et al., 1987; Shashar et al., 1994; Kushmaro et al., 1996; Banin et al., 2000; Frias-Lopez et al., 2002); however, their functional roles in relation to the coral host are virtually unknown. A variety of mutualistic benefits have been suggested, including fixation and passage to the coral host of nitrogen and carbon (Williams et al., 1987; Shashar et al., 1994; Cooney et al., 2002; Rohwer et al., 2002; Lesser et al., 2004), as well as other nutrients (Knowlton & Rohwer, 2003) and secondary metabolites such as antibiotics (Castillo et al., 2001). In addition, a recent study has shown that some types of bacteria may exclude undesirable microbial organisms. Mucus from healthy *Acropora palmata* was found to contain antibiotic-producing bacteria and inhibit growth of potentially pathogenic microbes (Ritchie, 2006). Growing recognition that microbial communities associated with corals represent an important component of coral symbioses highlights the need to better understand the nature and specificity of these microbial symbioses. Therefore, it is necessary to identify which bacteria are conserved as mutualistic partners and what factors may drive the structure of the coral microbial community, i.e. geography, environmental factors or differences in coral physiology.

Culture-independent techniques, in particular retrieval of 16S rRNA gene sequences, have enabled investigators to identify a wide range of microbial groups associated with corals. For example, Rohwer et al. (2002) examined three Caribbean species and found 430 distinct ribotypes from 14 coral samples. Additional studies examining other coral species from different geographic regions have noted similar diverse bacterial assemblages associated with corals (Rohwer et al., 2001; Friaz-Lopez et al., 2002; Bourne & Munn 2005). Though the abundance and types of bacterial groups have varied among coral species, there is also some evidence that the same coral species from geographically separate reef environments host the same bacterial communities (Rohwer et al., 2002). These findings support emerging generalizations that corals harbor species-specific bacterial communities that are geographically consistent. However, current studies have focused on corals from different families, raising questions about specificity at the level of coral species. Bacterial communities associated with corals have also been shown to be dynamic in response to environmental changes (Ritchie & Smith, 1995; Pantos et al., 2003; Ritchie, 2004; Barash et al., 2005; Koren & Rosenberg, 2006; Bourne et al. 2008), although the significance of shifts in microbial composition to coral health is currently unknown. There is a growing need to evaluate the taxonomic level and geographic extent to which bacterial communities are conserved on corals; in other words, whether coral species maintain specific bacterial consortia.

Greater knowledge of the bacterial communities associated with reef-building corals will aid in our understanding of this multi-species mutualism and will help to identify which species play a key role in maintaining coral health. This study aimed to examine coral-associated bacterial communities to test assumptions about specificity in coral-bacterial associations. We compare bacterial profiles recovered from three

closely related species of *Acropora*: *A. millepora*, *A. tenuis* and *A. valida* to determine whether genetically similar corals differ in the structure of their bacterial communities. Bacterial profiles were compared between two locations on the Great Barrier Reef (Magnetic and Orpheus Island reefs) to identify which bacteria might be conserved across geographically distinct locations. *A. millepora* samples were additionally collected throughout one year on Orpheus Island to investigate whether temporal environmental changes would lead to natural variation in coral bacterial community composition. Three culture-independent 16S rRNA gene profiling methods (clone library construction, denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism) were used to cross validate our findings and provide an accurate assessment of bacterial community composition.

2.2 Materials and Methods

2.2.1 Sample collection and processing

Three replicate healthy colonies of each of three coral species, i.e. *Acropora millepora*, *A. tenuis* and *A. valida,* were tagged on the reef flat (2 - 4 m depth) in Nelly Bay, Magnetic Island (19º10'S 146º50'E) and Pioneer Bay, Orpheus Island (18º35'S 146º29'E), Australia. Both are inshore, fringing reefs in the central Great Barrier Reef (GBR), though Magnetic Island reefs are more coastal and subjected to greater seasonal sea temperature variation, sedimentation and nutrients (Larcombe et al., 1995). All three coral species were sampled within the same transect at each site with replicate colonies of each species spaced 3-10 m apart to ensure colonies were subject to the same environmental factors. Coral branches were collected from the center of each colony and placed in plastic bags underwater. Samples were rinsed with sterile artificial sea water (ASW) to remove loosely associated microbes from the water

column, placed in new bags and frozen in liquid nitrogen for transport. Samples were later airbrushed (80psi) with 2ml of sterile ASW and the tissue slurry was aliquoted into two cryovials and stored at -80ºC. Samples used for species comparison were collected in May 2007 when seawater temperatures at the two sites were between 25-26ºC. In addition, replicate *A. millepora* colonies located on Orpheus Island were further sampled in July, October and December 2007 and February 2008 to assess temporal variability. Seawater temperatures were measured using data loggers positioned both at Orpheus Island and Magnetic Island and were associated with the Australian Institute of Marine Science long term monitoring data collection. Temperatures during this time ranged between 20°C in the Austral winter (beginning June) and 31°C in the summer (ending in February).

2.2.2 DNA extraction and purification

DNA was extracted by suspending 200 µl of coral tissue slurry in 0.5 ml of buffer (0.75M Sucrose, 40mM EDTA, 50mM Tris, pH 8.3) and following the extraction protocol outlined in Bourne et al. (2008). The DNA pellet was suspended in 30 µl of sterile Milli-Q water and the total volume was loaded on a 1.2% low-melting agarose gel. DNA was purified by using electrophoresis and cutting high quality DNA (>2kb) from the gel. The agarose was then removed from the sample by using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA was recovered from the Qiagen column with two 30 µl washes of sterile Milli-Q water.

2.2.3 PCR amplification of bacterial 16S ribosomal RNA genes

Bacterial specific primers 63f and 1387r (Marchesi et al., 1998) were used to amplify the 16S rRNA genes from extracted DNA for bacterial clone library

construction. Amplification of the 16S rRNA genes for terminal restriction length fragment polymorphism analysis was performed using the Beckman D4 labelled 63F primer and 1389R primer. The PCR mixtures (50ul) contained 0.2 pmol/ul of each primer, 200 mM each deoxynucleoside triphosphate, 1X PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5mM $MgCl_2$), 0.08% (w/v) bovine serum albumin and 1.25 U of *Taq* polymerase (Scientifix, Clayton, VIC, Australia). PCR was performed with an Applied Biosystems 2720 thermocycler and programmed with an initial 3 minute step at 94ºC and 35 cycles consisting of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1.5 minutes and a final extension for 10 minutes at 72ºC.

For denaturing gel electrophoresis, the bacterial 16S rRNA gene was amplified using primers 1055f and 1392R-GC (Ferris et al., 1996). PCR reactions (50µl) consisted of 0.5 µM of each primer, 100µM of each deoxyribonucleotide triphosphate, 0.08% (w/v) bovine serum albumin, 1X PCR buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 1.5mM $MgCl_2$), 1.5 mM $MgCl_2$ and 1.25 units Hotstar *Taq* (Qiagen). DGGE PCR reactions were carried out using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) thermocycler. Temperature cycling was performed using a touchdown protocol (Ferris et al., 1996) with one cycle of 95ºC for 15 minutes, 10 cycles of 94º for 1 minute, 53ºC (each cycle decreasing by 1ºC) for 1 minute, and 72ºC for 1 minute, followed by 20 cycles of 94ºC for 1 minute, 43ºC for 1 minute and 72ºC for 1 minute.

2.2.4 Clone library construction

The amplified bacterial DNA was ligated into the TOPO-TA cloning vector (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Ligations were submitted to the Australian Genome Research Facility for transformation, cloning

and subsequent sequencing. Ninety-six clones were sequenced from each library using the M13f primer.

2.2.5 Denaturing gradient gel electrophoresis analysis

Bacterial profiling was carried out using an INGENY phorU-2 (Ingeny International BV, Netherlands) DGGE system. PCR products were separated on gels containing 6.5% acrylamide with a 50-70% linear gradient of formamide and urea, using 0.5 x TAE buffer (0.02M Tris base, 0.01M sodium acetate and 0.5 mM Na_2 EDTA; pH adjusted to 7.4). The buffer was preheated to 60ºC prior to sample loading, and the electrophoresis run at 30v for 20 minutes to draw the DNA into the gel before running buffer through the system. Electrophoresis was then run at 60ºC for 16 hours at 70v. Gels were removed and stained for 10 minutes with SYBR Gold nucleic acid stain (Molecular Probes Inc., Eugene OR, USA) in 1X TAE buffer. Gels were de-stained by rinsing with 1XTAE buffer and subsequently photographed using a UV trans-illuminator.

Clear bands were excised from the gel and placed in 100 µl of sterile Milli-Q water to elute DNA from the acrylamide gel. The DNA bands were re-amplified and run on the DGGE gel to ensure correct migration and purity of the product. Products that showed one distinct band with the correct mobility on the DGGE were directly submitted for sequencing. Sequencing reactions were performed by Macrogen Inc. (Seoul, Korea) using the 1055F oligonucleotide as the sequencing primer.

2.2.6 Terminal restriction fragment length polymorphism analysis

Prior to restriction digestion, 3 replicate PCR products for each sample were purified using the QIAquik PCR Purification Kit (Qiagen, Hilden, Germany). The

restriction reaction mix was prepared with 1X NEB Buffer 4 (Scientifix, Clayton, VIC, Australia), 1 x BSA and 10U of Hha1 and added to 15 µl of PCR product. Samples were incubated at 37ºC for 5 hours and subsequently heat inactivated for 20 minutes at 65ºC. The digested DNA was precipitated from the solution by adding 2 µl of NaOAc (pH 5.2) and 50 µl of 95% ice-cold ethanol and centrifuged at 13,000 rpm for 5 minutes at 4ºC. The ethanol was removed and the DNA again washed with 100 µl of 70% ethanol. All ethanol was removed and the sample air dried until residual alcohol had evaporated. The DNA pellets were re-suspended in a solution containing 0.25 µl of size standard (600 bases) and 39.75 µl of SLS and loaded onto a 96-well sample plate. Each digest was then overlaid with 1 drop of mineral oil to avoid evaporation.

Digested samples were separated on a Beckman Coulter CEQ 8800 sequencer (Fullerton, CA, USA). Fragment analysis was performed on an 8 capillary array in fragment analysis mode using Beckman Coulter CEQ 8000 software (Fullerton, CA, USA). The following parameters were set: Modified fragment 4 injection time 20 seconds with a ramp to 2kV over 2 minutes and run at 4.9kV at 60°C for 60 minutes. Peak size and retention times were analyzed using a quartic model. The threshold for relative peak height was set at 20% of the height of the second highest peak to remove any spurious artifact peaks. Replicate samples were compared using T-align (Smith et al. 2006) with a range of 0.5 peak area to determine the consensus peaks between duplicates. The list of fragments for each sample was then converted into a binary matrix.

2.2.7 Sequence analysis

Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al., 1996). Sequence data were

aligned to the closest relative using the BLAST database algorithm (Altschul et al., 1997). Sequence affiliations were determined by >97% identity to bacterial 16S rRNA gene sequences in the GenBank database.

2.2.8 Statistical analysis

Statistical analyses were carried out using PAST statistical software (Ryan et al. 1995). T-RFLP profiles were examined using non-metric multidimensional scaling (NMDS) to determine whether sample profiles grouped according to species or location. An Euclidean similarity measure was used to generate a 2-dimensional plot. A principal components analysis (PCA) was used to analyze clone libraries to determine which dominant bacterial ribotypes contributed to the observed differences in coral bacterial community profiles. Any ribotype constituting 5% or more (arbitrarily assigned as dominant) of each clone library was included in a principal components analysis.

2.3 Results

2.3.1 Clone library analysis of bacterial diversity

The bacterial diversity associated with the three coral species, *A. millepora*, *A. tenuis* and *A. valida,* were highly similar for samples derived from the Magnetic Island site. Sequences from duplicate clone libraries of each species were consistent at the class level and dominated by γ-Proteobacteria, which represented approximately half of the sequences retrieved (Table 2.1). The second most abundant class was the α-Proteobacteria, which comprised between 10% and 33% of retrieved sequences, while a small proportion of clones affiliated within the δ-Proteobacteria class, constituting between 1-12% of the libraries (Table 2.1). Comparisons of identical coral species at a

second location (Orpheus Island) displayed similar microbial diversity profiles at the class level with both the α-and γ-Proteobacteria affiliated sequences dominating the libraries. The γ-Proteobacteria constituted between 18-40% of libraries while the α-Proteobacteria affiliated sequences represented 16-32% of all the libraries, except for one sample dominated by these sequences (71% of OItenuis-1 library). Unlike the Magnetic Island samples, β-Proteobacteria affiliated sequences comprised a large proportion of some of the Orpheus Island libraries (between 3-19% of sequences; Table 2.1).

Table 2.1 Proportions of bacterial taxonomic classes for each library

Bacteria classification	Magnetic Island clone libraries (%)						Orpheus Island clone libraries (%)					
	A.tenuis		***A.millepora***		***A.valida***		***A.tenuis***		***A.millepora***		***A.valida***	
	1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
α-proteobacterium	12	22.4	34.8	33	20	16.5	71.1	31.1	30.7	16.5	32.1	30.8
β-proteobacterium	0	1.2	1.1	0	0	1.3	3.3	0	14.7	19	9.4	17.3
δ-proteobacterium	2.2	1.2	9.8	12.1	1.3	1.3	0	0	0	0	0	0
γ-proteobacterium	58.7	58.8	40.2	49.5	67.5	54.4	17.8	40	37.3	31.6	32.1	38.5
Firmicutes	0	0	0	0	0	1.3	0	1.1	0	0	0	0
Bacteroidetes	0	0	0	0	0	0	0	1.1	1.3	0	0	1.9
Cyanobacteria	12	0	0	0	0	0	0	0	0	0	0	0
Unclassified	15.2	16.5	14.1	5.5	11.3	25.3	7.8	26.7	16	32.9	26.4	17.3

Investigation of the sequences retrieved from the clone libraries at the lower taxonomic levels of genera and family also displayed consistent microbial diversity within coral species at a particular site. Libraries derived from Magnetic Island corals were dominated by sequences affiliated with *Spongiobacter*, *Marinobacter*, *Acinetobacter*, *Roseobacter* and *Anaeromyxobacter* species, with the relative

proportion of ribotypes within these libraries similar for all coral species (Figure 2.1). In contrast, libraries derived from Orpheus Island corals were dominated by sequences affiliated with a different suite of bacterial genera, i.e. *Achromobacter*, *Brevundimonas* and *Caulobacterales* species. Similar to the bacterial communities associated with Magnetic Island corals, the relative proportion of the ribotypes within the Orpheus Island derived clone libraries were consistent for each coral species, the exception being *A. valida* derived libraries which additionally contained sequences affiliated with *Idiomarina* and *Flavobacteria* related organisms (Figure 2.1). Despite differences of many dominant groups within libraries from Magnetic and Orpheus Island corals, ribotypes affiliated with *Stenotrophomonas* species were consistent between libraries at the two sites. *Vibrio* affiliated sequences were also consistently retrieved from corals at both sites with the exception of the Orpheus Island *A. millepora* samples.

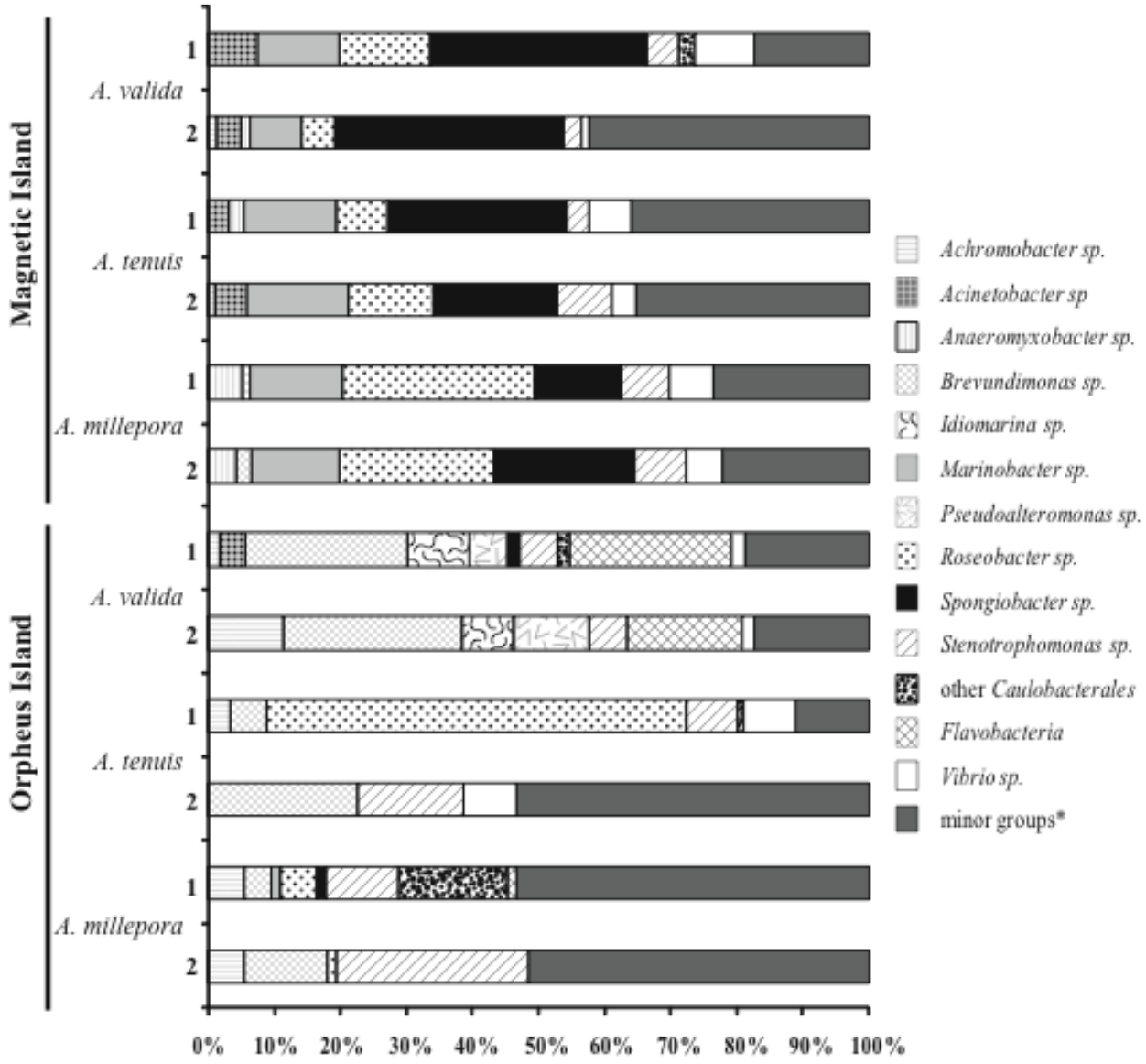

Figure 2.1 Dominant bacterial sequence affiliations for Magnetic and Orpheus Island clone libraries. Sequences were grouped into dominant ribotypes at the genera and class level. Only groups representing 5% or more of the clone libraries are represented in the figure. *this group represents sequences comprising less than 5% of each library and unclassified bacteria.

A principal components analysis (PCA) taking into account dominant clone types within each library (>5% of clones) displayed separation of libraries based on location, with the dominant bacterial ribotypes driving these differences (Figure 2.2). The first two principal components described 79% of the variation in the relative proportions of dominant bacterial ribotypes. Libraries derived from Magnetic Island

corals grouped together and correlated strongly with *Spongiobacter* and *Marinobacter* affiliated clones (Figure 2.2). Other sequences affiliated with *Acinetobacter, Vibrio, Spirulina* and *Anaeromyxobacter*, weakly correlated with the Magnetic Island samples. In contrast, Orpheus Island coral microbial communities appeared to be dominated by a different consortium of bacterial genera. Libraries derived from Orpheus Island corals correlated strongly with *Brevundimonas*, *Stenotrophomonas* and *Flavobacter* affiliated sequences and weakly with *Delftia, Idiomarina, Pseudoaltermonas,* and *Pseudomonas* affiliated sequences. *Roseobacter*-affiliated sequences were present in all libraries derived from Magnetic Island samples, constituting between 5-29% of sequences (Figure 2.1). Within the libraries derived from Orpheus Island corals, *Roseobacter*-affiliated sequences were only retrieved from *A. millepora* libraries at low levels (1-5% of sequences) but dominated the *A. tenuis* library 1 (63% of sequences retrieved). This resulted in a separation of this library from other tightly grouping Orpheus Island derived libraries in the PCA analysis (Figure 2.2).

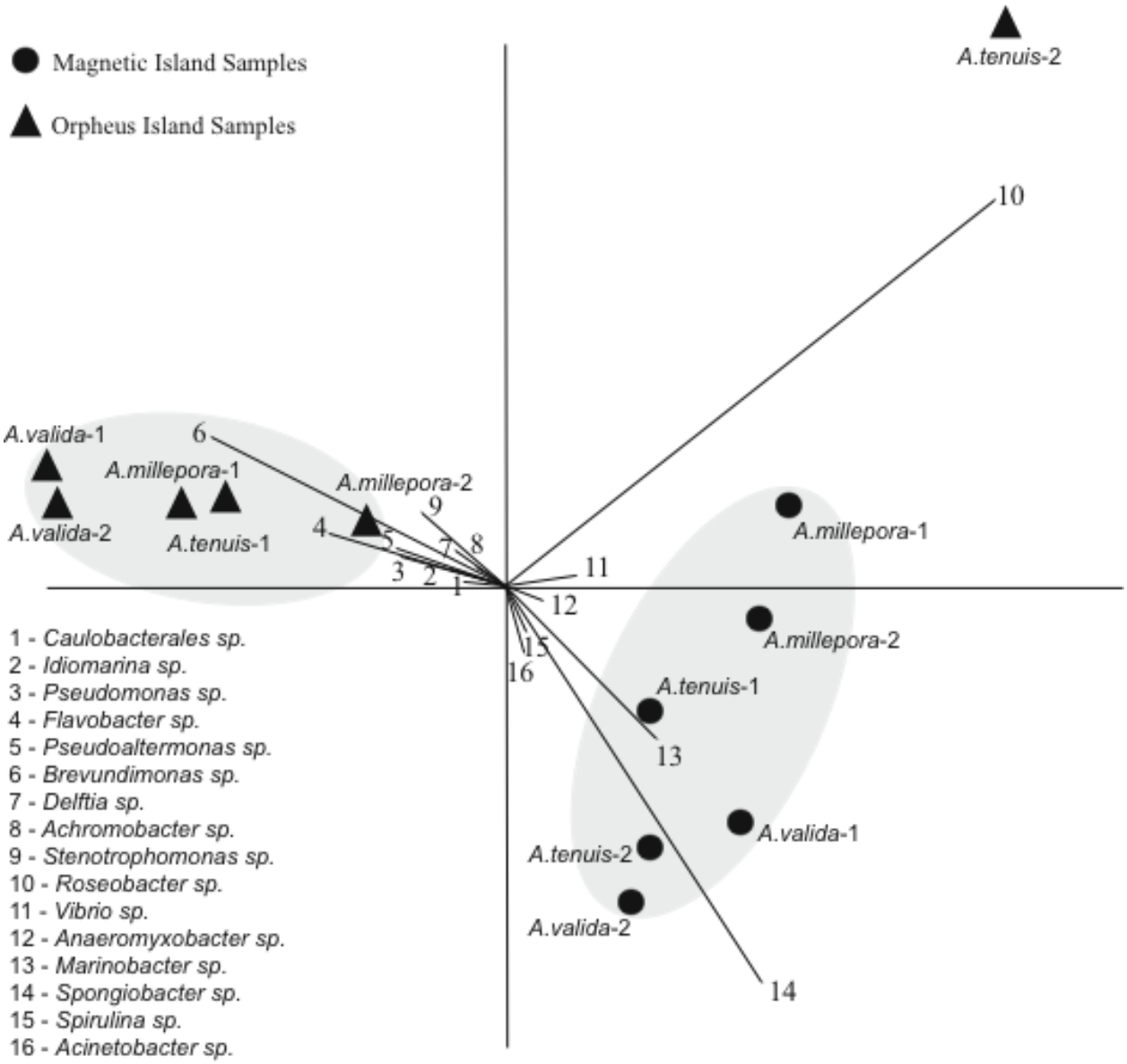

Figure 2.2 Principal components analysis of clone library sequences. Sequence affiliations included in analysis constituted >5% of each library.

A. millepora colonies located at Orpheus Island were again sampled in, July 2007 (winter) and February 2008 (summer) to compare seasonal changes in microbial diversity. The clone libraries were pooled since the relative abundance of ribotypes were consistent between duplicate samples derived from each sampling time point. Direct comparisons of the diversity patterns between winter (July) and summer (February) were similar (Figure 2.3). The dominant bacterial ribotypes in each library, which were also consistent with the previous sampling in May 2007 (Figure 2.1),

included *Brevundimonas* sp. (18.5%, 15.7%), *Achromobacter* sp. (7%, 1%) and *Stenotrophomonas* sp. (6%, 2%) for, July and February derived libraries respectively (Figure 2.2). Other dominant sequences consistent between summer and winter samples, such as *Idiomarina* sp. (7.6% and 11.8%), *Pseudoaltermonas* sp. (12% and 16.7%) and *Vibrio* sp. (17.4% and 13.7%), were not however retrieved from May samples. Aside from *Shigella*-like species recovered from July libraries, all other less abundant ribotypes were isolated from libraries from winter and summer time periods, indicating that temporal changes had little impact on the bacterial communities of *A. millepora* on Orpheus Island despite large temperature shifts between winter (July average daily seawater temperature 20°C) and summer (February average daily seawater temperature 30°C). A summary and comparison of all sequences retrieved from all libraries is presented in Appendix 1 along with the closest affiliated sequence in the GenBank database under accession numbers FJ489682-FJ489837.

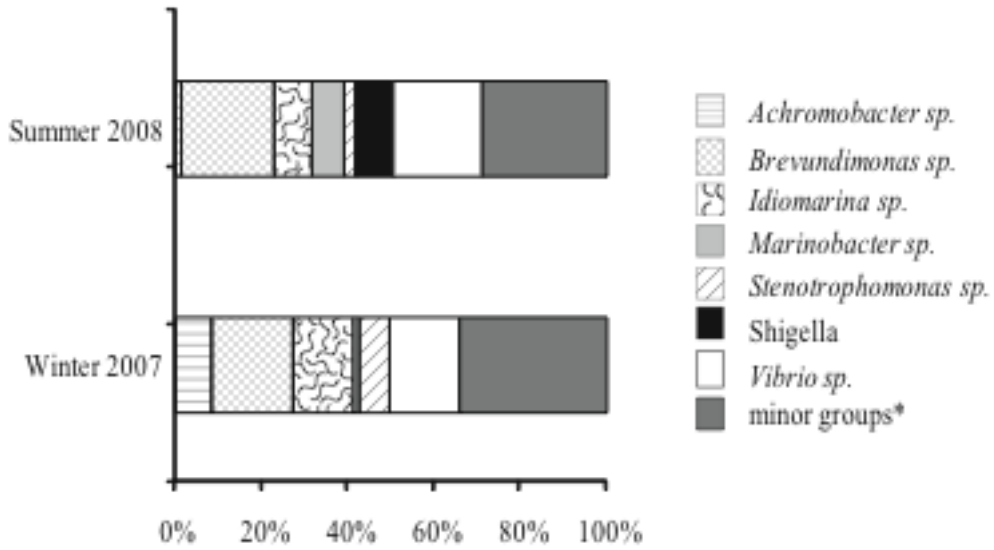

Figure 2.3 Dominant bacterial sequence affiliations for summer (February 2008) and winter (July 2007) Orpheus Island clone libraries. Sequences were grouped into dominant ribotypes at the genera and class level. Only groups representing 5% or more of the clone libraries are represented in the figure. *this group represents sequences comprising less than 5% of each library and unclassified bacteria.

2.3.2 Bacterial fingerprinting of coral associated microbial communities

Bacterial community profiles of coral species assessed by denaturant gradient gel electrophoresis (DGGE) displayed almost identical banding patterns for replicate samples of the same coral species (Figure 2.4). These profiles were also similar between coral species located from the same reef site (Magnetic Island or Orpheus Island), suggesting a high level of similarity between dominant bacterial ribotypes associated with these three species of *Acropora*. Though DGGE bacterial profiles were similar between replicates and species within sites, the banding patterns differed between colonies located around Orpheus Island and Magnetic Island. The sequences retrieved from Magnetic Island profiles affiliated with *Roseobacter*, *Anaeromyxobacter*, *Pseudomonas*, *Spongiobacter*, *Vibrio,* and *Marinobacter* species (Table 2.2). Sequences isolated from Orpheus Island corals affiliated with *Brevundimonas*, *Achromobacter* and *Serratia marcescens* related organisms. The retrieved sequences from DGGE analysis of the respective corals at each location matched the dominant ribotypes recovered from clone libraries derived from these sites, cross-validating our findings. DGGE bacterial fingerprints of *A. millepora* corals repeatedly sampled from Orpheus Island throughout the year, showed nearly identical profiles between replicate samples and for each month examined (Figure 2.5). The sequences of dominant bands (Table 2.2) were again consistent with dominant ribotypes in the clone libraries and provide further evidence of consistent bacterial communities associated with corals despite temporal temperature changes.

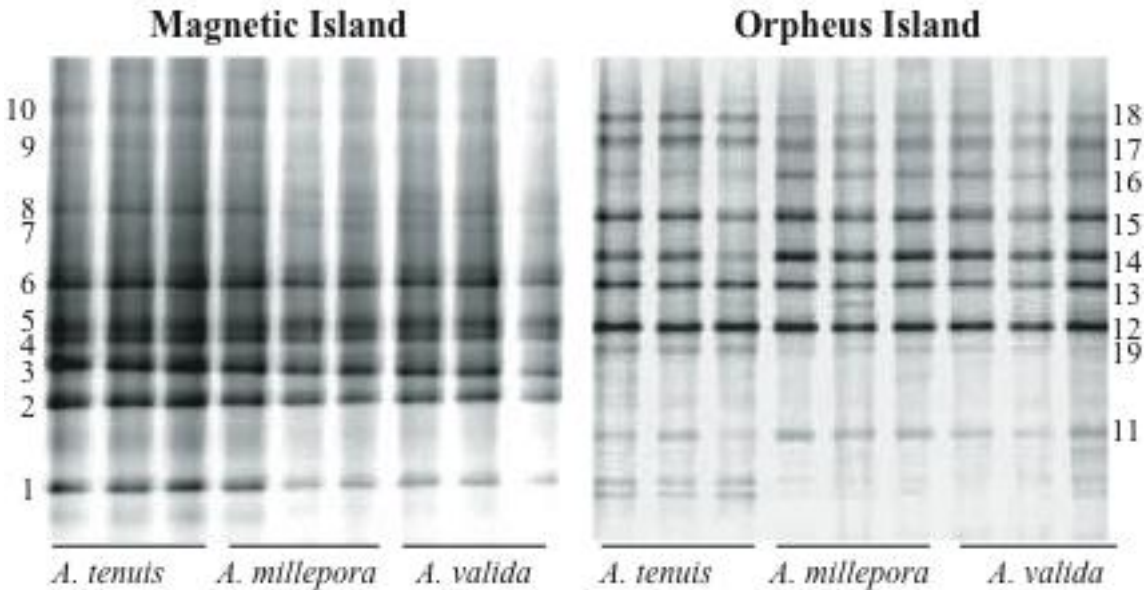

Figure 2.4 Denaturing gradient gel electrophoresis for Magnetic and Orpheus Island coral samples. Band numbers correspond to sequences retrieved in Table 2.

Table 2.2 Affiliation of DGGE bacterial sequences retrieved from coral colonies for Magnetic Island (n=9) and Orpheus Island (n=9)

Band No.	Closest relative and database accession number	Alignment (bp)	Similarity* (%)	Taxonomic description
Magnetic Island				
Band 1	*Roseobacter* sp. (DQ412059.1)	277/281	98	α-Proteobacteria
Band 2	*Anaeromyxobacter dehalogenans* (EU331403.1)	302/307	98	δ-Proteobacteria
Band 3	*Marinobacter hydrocarbonoclasticus* (EU624424.1)	303/308	98	γ -Proteobacteria
Band 4	*Pseudomonas* sp. (EU770402.1)	303/309	99	γ-Proteobacteria
Band 5	*Spongiobacter nickelotolerans* (AB205011.1)	297/312	95	γ-Proteobacteria
Band 6	Uncultured bacterium (EU628068.1)	309/312	99	
Band 7	*Vibrio* sp. (EU143360.1)	272/277	98	γ-Proteobacteria
Band 8	*Pseudomonas* sp. (EU770402.1)	277/281	98	γ-Proteobacteria
Band 9	*gamma proteobacterium* (DQ351795.1)	309/312	99	γ-Proteobacteria
Band 10	*Spongiobacter nickelotolerans* (AB205011.1)	305/313	97	γ-Proteobacteria
Orpheus Island				

Band 11	*Muricauda aquimarina* (AY445076.1)	301/308	97	*Bacteroidetes*
Band 12	Uncultured bacterium (EU540610.1)	296/299	98	
Band 13	*Achromobacter xylosoxidans* (EU827475.1)	287/290	98	β-Proteobacteria
Band 14	*Achromobacter xylosoxidans* (EU827475.1)	277/287	96	β-Proteobacteria
Band 15	*Brevundimonas* sp. (AB447548.1)	265/269	98	α-Proteobacteria
Band 16	*Achromobacter xylosoxidans* (EU308119.1)	277/287	96	β-Proteobacteria
Band 17	Uncultured bacterium (AJ428146.1|UBA428146)	296/299	98	
Band 18	*Serratia marcescens* (FM163485.2)	287/290	98	γ-Proteobacteria
Band 19	Uncultured bacterium (EU540603.1)	293/298	98	
Band 20	*gamma proteobacterium* (AY868014.1)	293/299	99	γ-Proteobacteria

* Sequences were aligned to the closest relative using BLAST (Altschul, 1997). The similarity was calculated with gaps not taken into account

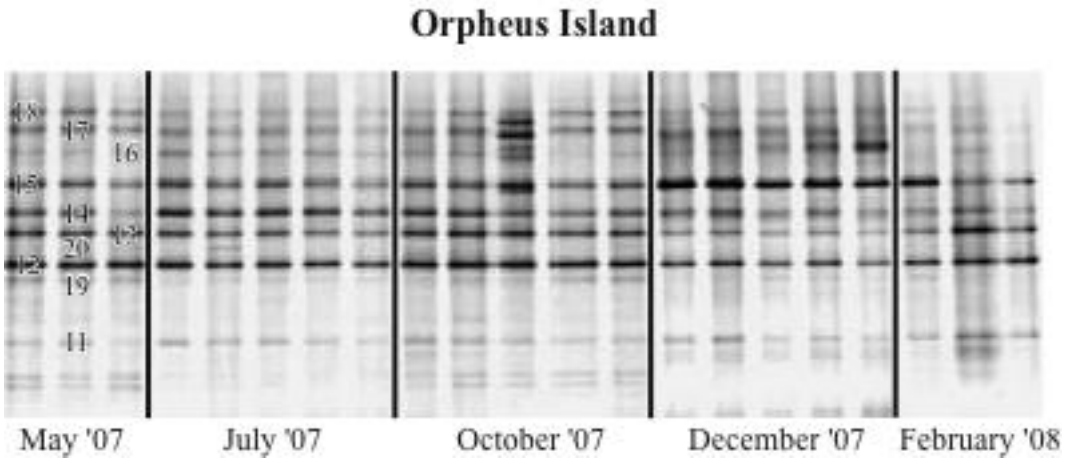

Figure 2.5 Denaturing gradient gel electrophoresis for Orpheus Island samples collected from May 2007-February 2008. Band numbers correspond to sequences retrieved in Table 2.

Comparisons of terminal restriction length polymorphism (t-RFLP) patterns of replicate samples from each coral species at each site were consistent. Peak profile patterns (based on presence or absence) incorporated into a non-metric multi-dimensional scaling (nMDS) analysis demonstrated grouping of the coral bacterial patterns within locations (Figure 2.6). Similar to clone library and DGGE analysis, bacterial diversity patterns of the closely related coral species were also similar and

clustered together in the nMDS analysis (Figure 2.6). Although profiles derived from Magnetic Island samples were not tightly clustered, they were separated from profiles derived from Orpheus Island samples.

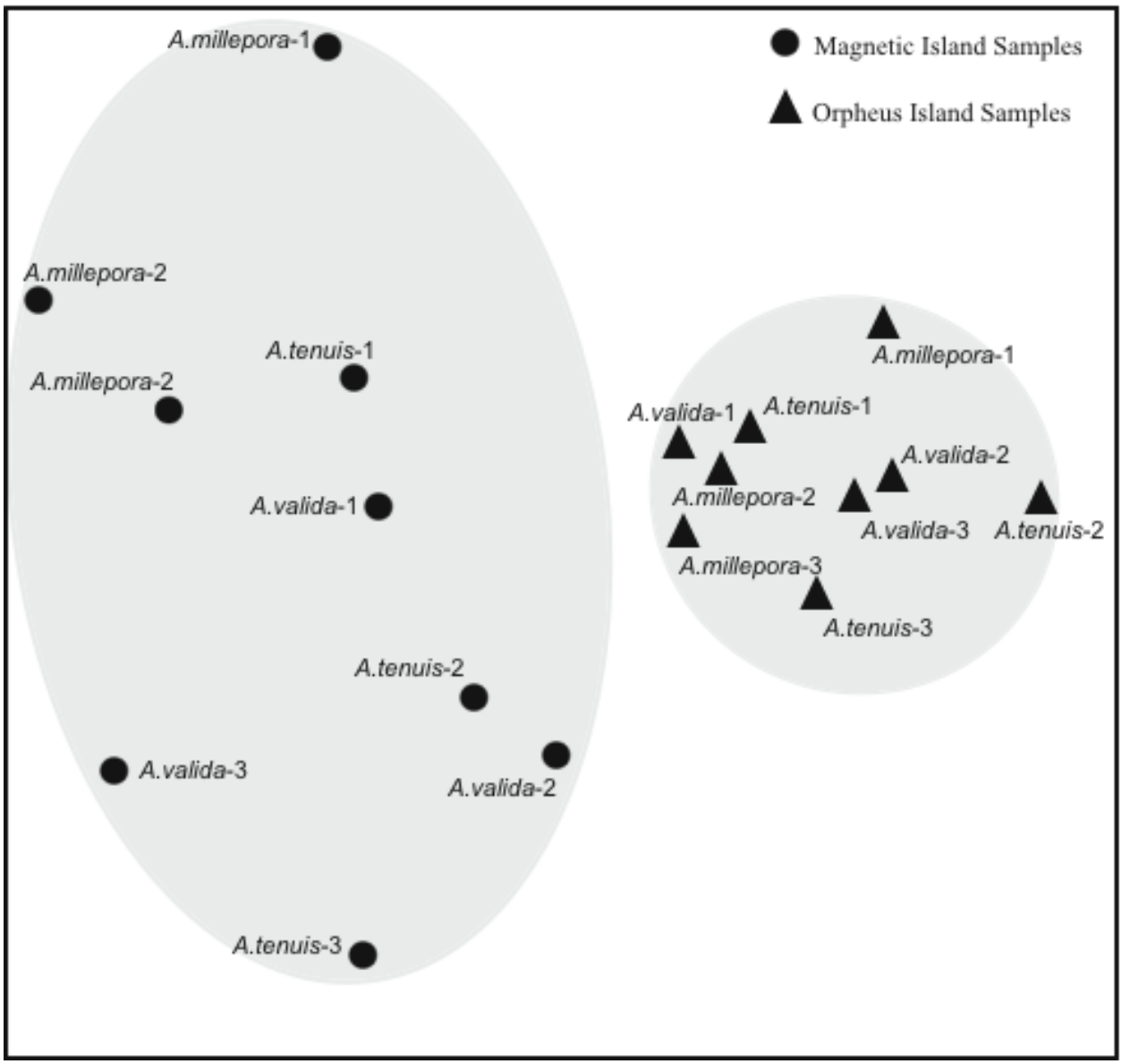

Figure 2.6 Representation of non-metric multi-dimensional scaling (nMDS) plot of terminal-restriction fragment length polymorphism (t-RFLP) profiles. The data was obtained by distance matrix analyses of t-RFLP fingerprints.

2.4 Discussion

Previous studies have highlighted high bacterial diversity associated with corals but have also suggested that some bacteria form relatively stable and species-specific

associations (Rohwer et al., 2001; Friaz-Lopez et al., 2002; Bourne & Munn, 2005). Through poorly understood complex interactions, corals structure these microbial partners which are suspected to play an important role in the coral host's health (Reshef et al., 2006). Observations of conserved communities have contributed to the coral holobiont principle, the concept of a structured symbiosis between the coral host and its microbial partners (Knowlton & Rohwer, 2003; Zilber-Rosenberg & Rosenberg, 2008). This study investigated the principle of conserved bacterial communities associated with three closely related species of *Acropora* and demonstrated that not only were the bacterial profiles of replicate coral samples of the same species highly similar, but microbiota of closely related species within the same site were also conserved. These conclusions were based on consistent observations in dominant bacterial ribotypes recovered from clone libraries of each species within the locations as well as consistent DGGE and t-RFLP profiling fingerprints.

Within the current study, we were particularly interested in the dominant ribotypes and sequences retrieved from the respective 16S rRNA profiling techniques. For clone libraries, ribotypes representing greater than 5% of each library were used as a cut-off to indicate a dominant member of the microbial community. Using this principle of dominant clone types within the libraries, a PCA plot indicated that the bacterial communities associated with corals at two sites separated by 40 km, an inshore fringing reef (Magnetic Island) and a coastal reef (Orpheus Island), displayed different microbial diversity patterns. NMDS analysis of bacterial t-RFLP profiles and DGGE fingerprints supported this observation. T-RFLP profiles, like clone libraries, were selected based on a threshold for relative peak height to account for dominant sequences within duplicate PCR's, while DGGE is known for providing only dominant profiles within a community (Gelsomino et al., 1999; Maarit Niemi et al., 2001;

Muyzer & Smalla, 1998; Muyzer et al., 1993; Nikolausz et al., 2005). Sequences retrieved from both DGGE and clone library analyses were consistent, supporting and cross-validating the conclusions that differences in the dominant bacterial community members exist on corals located at the two reef systems.

Previous studies have reported that similar bacterial populations have been found on the same coral species that are geographically separated (Rohwer et al., 2001, 2002), suggesting that corals associate with certain bacteria despite geographic location. While the current study demonstrates that bacterial profiles separate corals based on geographic location, these conclusions are based on the dominant profiles driving the differences. When we look at individual sequences which represent minor components of retrieved 16S rRNA sequences from clone libraries, we see consistent sequences between the corals (Appendix 1). For example, *Roseobacter* and *Stenotrophomonas* affiliated sequences are observed in all libraries. While *Spongiobacteria* affiliated sequences are dominant in the Magnetic Island samples (12-27% within all libraries), they are also retrieved in low relative number in the Orpheus Island clone libraries (1%). This indicates that similar phylotypes are associated with corals and the multitude of coral diversity reports in the last ten years similarly report common coral associated bacteria (Cooney et al., 2002; Rohwer et al. 2002; Bourne & Munn, 2005). The observations in the current study indicate that while the same bacterial ribotypes are observed within all the corals investigated, dominant members of the community differ between location. However, what drives this apparent difference is unknown.

Reshef et al., (2006) proposed the coral probiotic hypothesis, which states that coral potentially adapt to new environmental conditions by altering their specific symbiotic bacterial partners. Environmental variables such as water quality, light

exposure or temperature may be determining factors driving the difference in dominant coral-associated bacteria at these two similar shallow in-shore reef environments on the GBR. Klaus et al. (2007) demonstrated that water depth in varying proximity to coastal pollution can affect the bacterial composition on healthy colonies of *Montastraea annularis*. While both coral reefs are proximally located to the shore, Orpheus Island is not inhabited, apart from a small research station, while Magnetic Island is populated and suffers some land discharge and pollution onto the reef (Larcomb et al. 1995; Muslim & Jones 2003). Factors such as light intensity (Kuta and Richardson, 2002; Richardson and Kuta, 2003), water temperature (Ben-Haim et al. 2003, Bruno et al. 2007, Gil-Agudelo and Garzon-Ferreira, 2001; Kuta and Richardson, 2002; Cervino et al., 2004; Rosenberg and Falkovitz, 2004) and increased pollution (Bruckner et al.,1997; Kim and Harvell, 2002; Patterson et al., 2002; Kaczmarsky et al., 2005) have all been correlated with shifts in coral microbial associations that lead to coral disease outbreaks. However, all corals in this study were visibly healthy at the time of sampling. It is unlikely that bacteria within the water column are driving the observable differences in the dominant bacteria communities since sequences affiliated with *Spongiobacter*, *Roseobacter* and *Stenotrophomonas* to name only a few, were a component of coral clone libraries at both locations, suggesting that many of these bacteria are present in both environments. One aspect of the coral holobiont that should be investigated is the potential for the photosynthetic dinoflagellate *Symbiodiunium* to drive differences in associated bacteria communities. This algal partner provides the coral host with the majority of its photosynthate nutrient sources, with different genotypes harboured by coral from separate locations (Baker, 2003, Berkelmans & van Oppen 2006). Variation in the photosynthetic activity, producing variable bio-available nutrient sources, may influence the

composition of coral mucus and thus indirectly the coral microbiota (Klause et al. 2007).

Previous studies have suggested that bacterial communities may be dynamic in healthy corals in response to seasonal temperature fluctuations. For example, Koren and Rosenberg (2006) examined the bacteria associated with *Oculina patagonica* in the summer and winter to establish the temporal changes in bacteria on healthy corals. While the dominant group (35% of sequenced clones) was *Vibrio splendidus* in both summer and winter, the next 10 most abundant clusters of bacteria differed between seasons and temperature was one factor identified as causing these microbiota shifts. Results from this study demonstrate that the dominant members of the bacterial community associated with *A. millepora* were relatively stable throughout the seasons on Orpheus Island despite temperature fluctuations of greater than 10°C. While there were differences in some dominant sequences retrieved from May sample libraries, DGGE profiles were consistent for all sampling periods and direct comparisons between libraries from winter (July) and summer (February) showed highly similar abundance of dominant ribotypes. The seasonal conditions are less variable along the GBR compared to the 20°C fluctuation in the Mediterranean Sea, which may provide one explanation for the observed differences by Koren and Rosenberg (2006) and the stable microbial communities observed associated with *A. millepora* within one site and throughout one year.

Magnetic Island corals displayed highly similar bacterial profiles when comparing duplicate libraries of the same species. While duplicate samples derived from Orpheus Island were similar, there were some differences particularly within the *A. tenuis* libraries, indicating within-colony variation, or patchiness of microbial associates. This is consistent with results from Rohwer et al. (2002), which

demonstrated that one bacterial ribotype resided only in the branch tip but not in other portions of *Porites furcata*. Furthermore, Bourne et al. (2005) showed that different microenvironments exist within the layers of *Pocillopora damicornis* in which the associated bacterial communities differed. Over-representation of retrieved *Roseobacter* sp. affiliated sequences in the *A. tenuis* library 1 caused separation in PCA analysis of dominant ribotypes within the libraries. This result highlights the potential for bias in PCR based bacterial profiling techniques, also indicating the strength of cross-validating the conclusions by using three different profiling techniques, each with different bacterial targeted PCR primer sets.

For corals located at Magnetic Island, sequences related to *Spongiobacter* species were of particular interest. These related sequences have been derived as the name suggests from sponges, but have also previously been observed as dominating culture-independent assessment of *Acropora millepora* coral species at Magnetic Island (Bourne et al., 2008). Bourne et al. (2008) showed that *Spongiobacter* sp. related sequences dominated bacterial profiles before and after a bleaching event, shifting away from this stable microbial associating during the stress period. Within the libraries of this study, *Spongiobacter* related sequences accounted for between 17-35% of clone libraries and was the dominant clone type. Being such a dominant member of the coral-associated microbial community indicates that *Spongiobacter* play a key role in the holobiont functioning system, though at present little is known what role this may be. Other sequences dominating libraries for coral at Magnetic Island such as *Roseobacter* (9-38%), *Stenotrophomonas* (4-13%), and *Marinobacter* (17-24%) species also potentially perform necessary functions. For instance, *Marinobacter* sp. are aerobic halophilic bacteria that can utilize a range of carbon sources as well as degrade hydrocarbons (Marquez & Ventosa, 1995; Huu et al., 1999;

Nicholson & Fathepure, 2004) which may be vital to the carbon cycle within the holobiont. The bacterial inhabitants of corals from Orpheus Island that comprised the greatest portion of the clone libraries, ie. *Brevundimonas* sp. (4-22%), *Stenotrophomonas* sp. (8-26%), and *Roseobacter* sp. (5-38%) are possible candidates for investigation in coral nutrient cycling or production of antimicrobial properties. For example, *Brevundimonas* sp. and other *Caulobacterales* are anaerobes found ubiquitously in water and are believed to have important roles in carbon cycling within their respective habitats (Staley, 1987; Abraham et al., 1999) and therefore may have a function in the host's nutrition. The marine *Roseobacter* clade comprises several genera of marine bacteria with heterogeneous physiological properties including sulfite reduction (Sorokin, 1995), metabolism of dimethylsulfoniopropionate (Zubkov et al. 2001, 2002), production of toxins (Gallacher et al. 1997, Hold et al. 2001) and aerobic anoxygenic photosynthesis (Algaier et al 2003). As such, *Roseobacter* species are suspected to have important functional activities within the coral holobiont, including the ability to produce secondary metabolites (Lafay et al., 1995; Gram et al., 2002). Some species have been found to produce antagonistic activity against other marine bacteria and algae (Brinkoff et al., 2004; Roa et al., 2005), which may also serve as a protection mechanism in coral excluding potential pathogenic organisms (Ritchie et al. 2006).

2.4.1 Unresolved Questions

As we begin to unravel what the players are in coral microbial communities, several questions arise as to what implication these associations have for coral health. Future studies need to explore what the primary holobiont factors are that may drive these consistent microbial associations and how environmental factors such as temperature, light and eutrophication can influence and subsequently change the

microbial consortia. An understanding of the fragility of these microbial associations and how their function in the holobiont supports coral health is essential to predict and understand what effect environmental change will have on coral health. Determining the functional roles of bacteria as well as other microbes such as *Archaea*, the endolithic community, algae and protozoans in coral associations will also allow us to distinguish whether there are symbiotic relationships between the coral host and certain microbes. In addition, we can determine whether there are a consortia of many bacteria interacting synergistically to form a multispecies partnership with the host, or whether there are bacteria merely taking advantage of the host as a suitable habitat without any reciprocated benefit.

Chapter 3.0 Bacteria associated with translocated corals differ from those associated with wild corals

Littman, R. A.; Bay, L.; Willis, B., Bourne, D.

3.1 Introduction

Corals form complex associations with bacteria and other microbial communities, including the key photosynthetic dinoflagellate partner *Symbiodinium*, plus *Archaea*, fungi and endolithic algae. Investigations of microbial communities associated with corals have provided insights into the functional roles these microbial constituents play in the coral holobiont (Rohwer et al. 2002). Major putative roles for bacterial associates include pathogen resistance and biogeochemical cycling within corals (reviewed in Mouchka et al. 2010). For instance, mucus from *Acropora palmata* was found to harbour antibiotic-producing bacteria that were capable of inhibiting the growth of potentially pathogenic microorganisms (Ritchie, 2006). Given the varied and essential roles bacterial constituents of the coral symbiosis are likely to play, it is important to determine what factors influence the composition of bacterial communities within the coral microbial community.

Bacteria associated with corals are known to be highly diverse. For example, Rohwer et al. (2002) estimated that 6,000 different bacterial ribotypes were present in 14 coral samples from three species of corals. Over 8,500 OTUs were detected among 7 different species of corals using pyrosequencing (Sunagawa et al. 2010). A number of other studies have found similar levels of species richness (Rohwer et al. 2001; Bourne and Munn 2005; Klaus et al. 2005; Koren and Rosenberg 2006; Sekar et al. 2006; Kapley et al.2007; Wegley et al. 2007; Koren and Rosenberg 2008; Lampert et al. 2008; Hong et al. 2009; Littman et al. 2009; Reis et al. 2009), highlighting the highly diverse and complex nature of these coral-bacterial associations. Early studies of spatial patterns of bacterial diversity associated with corals suggested that these associations are stable for corals within the same reef system, as well as for corals across different regions (Rohwer et al. 2001, Rohwer et al. 2002). Also, bacterial

composition is typically similar among adjacent corals of the same species, but differs from that in the water column (Frias-Lopez et al. 2002; Rohwer et al. 2002; Bourne and Munn 2005; Pantos and Bythell 2006), indicating that certain bacterial interactions may be maintained for the health of the coral holobiont. However, more recent studies have shown differences in bacterial communities associated with corals of the same species among locations. In particular, dominant bacterial ribotypes have been found to differ on corals from different geographical locations (Klaus et al. 2005, Guppy et al. 2006, Littman et al 2009, Kvennefors et al. 2010). For example, Littman et al. (2009) found consistent members of the microbial community on corals from two different reef locations, but the dominant bacterial sequences differed between the two sites (see Chapter 2). Hong et al. (2009) found variation in the bacterial associates of *Stylophora pistillata* within and between locations over time. These studies raise questions about what factors drive regional differences in coral-associated bacteria, particularly whether environmental factors play a role in determining the bacteria that corals host.

One fundamental question concerns the stability of bacterial communities hosted by corals. Some environmental factors have been shown to affect the composition of coral-associated bacteria. For example, Koren and Rosenberg (2006) compared bacteria associated with *Oculina patagonica* in summer and winter and found that, while the dominant group remained *Vibrio splendidus*, the other ten most abundant clusters of bacteria differed completely between seasons. Klaus et al. (2005) noted differences in bacterial profiles along gradients of pollution and water depth. Furthermore, temperature stress has been found to alter bacterial communities associated with *Acropora millepora*, the net result being a proliferation of *Vibrio* spp.

(Bourne et al. 2008). Thus, bacterial associations with corals may be highly dynamic, with various environmental factors causing changes in their community structure.

In this study, coral colonies were reciprocally translocated between a southern, cooler reef (Great Keppel Is) and a more northern, warmer reef (Magnetic Is) to determine if environmental differences associated with a changed geographical location would affect the structure of bacterial communities. A reciprocal design was used to distinguish between two alternative hypotheses, i.e. whether environmental factors determine the bacteria associated with corals, or whether genetic differences in coral hosts originating from the two sites determine the bacterial community. I demonstrate that the process of translocating corals changed the composition of the bacterial associates, indicating that environmental factors influence the dynamics of coral-associated bacteria.

3.2 Materials and methods

3.2.1 Reciprocal translocation of corals

The common Indo-Pacific coral *Acropora millepora* was reciprocally translocated between two locations on the Great Barrier Reef. In 2005, three years prior to translocation, 10 corals sourced from an inshore bay in the central GBR (Magnetic Island) and 20 colonies sourced from a southern inshore reef (Great Keppel Island) were placed on metal mesh racks at their approximate depth of origin (3m). In April 2008, the corals were sampled and divided into two. One half of each colony was placed on a rack at the site of origin and the other half was transported to the second site. Samples collected in April are referred to as 4K, indicating samples collected in the fourth month from Great Keppel Island, or 4M, indicating samples collected in the fourth month from Magnetic Island. Corals originating from Great Keppel Island and

transplanted to Great Keppel Island were labeled KK; corals originating from and kept at Magnetic Island were named MM. Those originating on Great Keppel Island and transplanted to Magnetic Island are referred to as KM, and those transplanted onto Great Keppel and originating on Magnetic Island are referred to as MK. Each coral was sampled every two months throughout the year until December 2008. Samples collected in April provided a common baseline sample for translocated and non-translocated (control) samples collected throughout the year. Five replicate coral colonies were also sampled on reefs near the racks to compare bacterial communities associated with 'wild' corals to those associated with translocated corals, to determine if experimental manipulation affected the bacterial community structure.

3.2.2 DNA extraction and purification

Coral nubbins were airbrushed and stored at -80°C until analysed. DNA was extracted from the coral slurry in 0.5 ml of buffer (0.75M Sucrose, 40mM EDTA, 50mM Tris, pH 8.3), following the extraction protocol outlined in Bourne et al. (2008). The DNA pellet was suspended in 30 µl of sterile milli-Q water and the total volume was loaded on a 1.2% low-melting agarose gel. DNA was purified by using electrophoresis and cutting high quality DNA (>2kb) from the gel. The agarose was then removed from the sample by using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA was recovered from the Qiagen column with two 30 µl washes of sterile milli-Q water.

3.2.3 PCR amplification of 16S ribosomal RNA gene

Universal primers 63f and 1387r (Marchesi et al. 1998) were used to amplify the 16S rRNA genes from extracted DNA for bacterial clone library construction. The PCR mixtures were prepared as outlined in section 2.2.3. PCR was performed with an

Applied Biosystems 2720 thermocycler, and programmed with an initial 3 min step at 94ºC, thirty-five cycles consisting of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1.5 minutes, and a final extension for 10 minutes at 72ºC.

For denaturing gel electrophoresis, the bacterial 16S rRNA gene was amplified using primers 1055f and 1392R-GC (Ferris et al. 1996). PCR reactions (50) were prepared as stated in section 2.2.3. DGGE PCR reactions were carried out using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) thermocycler. Temperature cycling was performed using a touchdown protocol (Ferris et al. 1996).

3.2.4 Clone library construction

The amplified bacterial DNA was ligated into TOPO®-cloning vector following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Ligations were submitted to the Australian Genome Research Facility for transformation, cloning and subsequent sequencing. Two replicate libraries of 96 clones each were sequenced for samples collected: before transplantation in April, during the cold water bleaching at Great Keppel Island in October, and during the warm water bleaching at Magnetic Island in December.

3.2.5 Denaturing gradient gel electrophoresis analysis (DGGE)

Bacterial profiling was carried out using an INGENY phorU-2 (Ingeny International BV, Netherlands) DGGE system, as outlined in section 2.2.5. Clear bands were excised from gels and placed in 100 µl of sterile milli-Q water to elute DNA from the acrylamide gel. The DNA bands were re-amplified and run on the DGGE gel to ensure correct migration and purity of the product. Products that showed one distinct band with the correct mobility on the DGGE were directly sequenced.

3.2.6 Sequence analysis

Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al. 1996). Sequence data were aligned to the closest relative using the Greengenes database (DeSantis et al. 2006). Sequence affiliations were determined by >97% identity to bacterial 16S rRNA gene sequences in the NCBI database.

3.2.7 Statistical analysis

Statistical analyses were carried out using PAST statistical software (Ryan et al. 1995). DGGE profiles were converted into a presence/absence matrix and were examined using non-metric multidimensional scaling (NMDS) to determine whether sample profiles grouped according to reef location. A Euclidean similarity measure was used to generate a 2-dimensional plot. A principal components analysis (PCA) was used to analyze clone libraries to determine which dominant bacterial ribotypes contributed to the observed differences in coral bacterial community profiles.

3.3 Results

3.3.1 Rack corals differ from wild corals

Clone libraries constructed for *A. millepora* colonies attached to racks show that they all associated with similar OTUs (Figure 3.1). This pattern held for: 1) corals prior to translocation at both sites (April 2008: 4K1, 4K2, 4M1, 4M2), 2) control and translocated corals maintained at Great Keppel Island (October 2008: 10KK1, 10KK2, 10MK1, 10MK2), and 3) control and translocated corals maintained at Magnetic Island (December 2008: 12MM1, 12MM2, 12KM1, 12KM2). Comparison of these clone libraries with ones previously constructed for wild Magnetic Island corals (Magnetic 1,

Magnetic 2; Chapter 2) illustrates that many of the bacterial OTUs associated with control and translocated corals did not occur on 'wild' corals at Magnetic Island, indicating that attaching corals to racks altered their bacterial communities. The dominant bacterial sequences found for rack corals were affiliated with *Vibrio* sp. (21.6%-49.1%), *Diaphorobacter* sp. (8.5%-25.5%), *Acidobacteria* sp. (3.5%-19.1%) and *Plesiocystis* sp. (2.0%-14.6%). For Magnetic Island 'wild' corals, the predominant sequences recovered were related to *Roseobacter* sp. (26.9% and 29.3%), *Spongiobacter* sp. (13.0% and 24.4%) and *Stenotrophomonas* sp. (7.6% and 9.0%). Only *Stenotrophomonas* sp.-affiliated sequences and *Vibrio* sp.-affiliated sequences are shared by corals on racks and 'wild' corals.

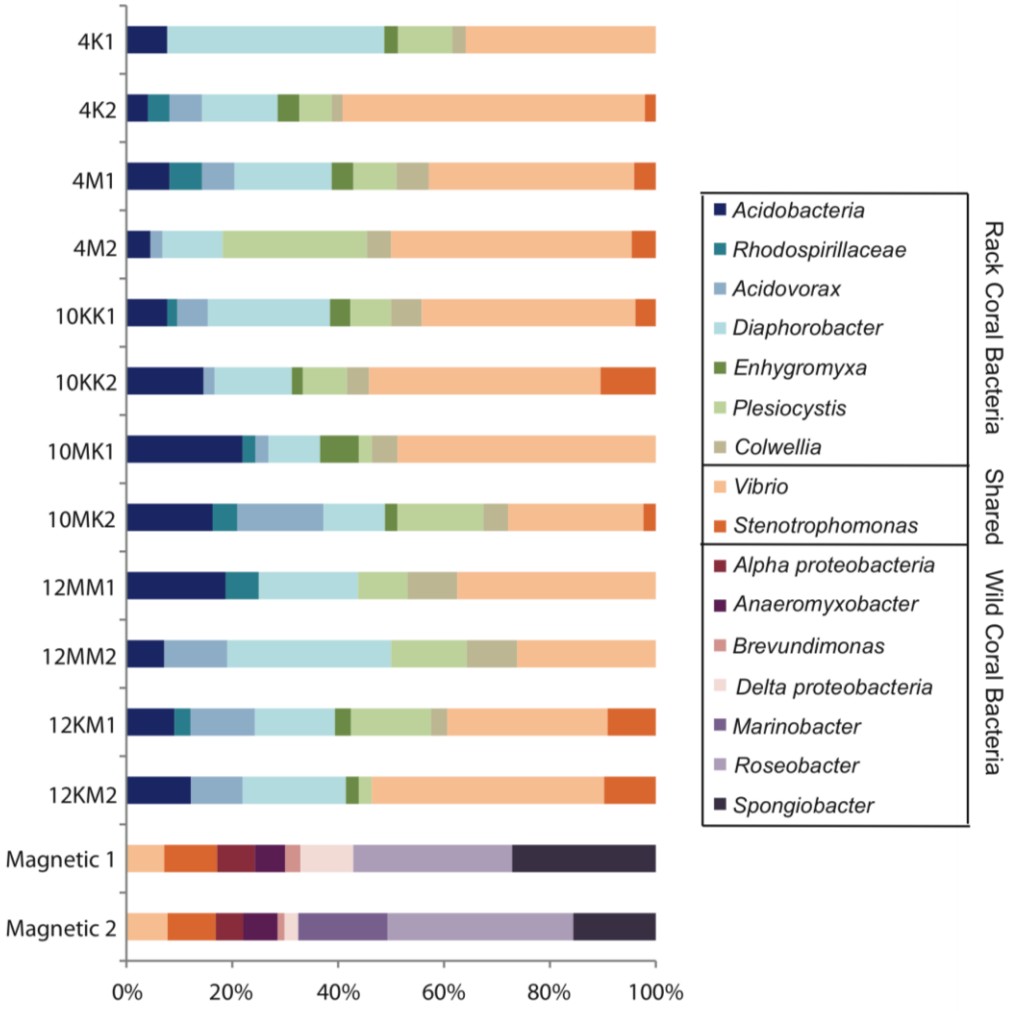

Figure 3.1 Comparison of clone library profiles for: 1) Great Keppel Island corals attached to racks (collected in April 2008; 4K1, 4K2), 2) Magnetic Island corals attached to racks (collected in April 2008; 4M1, 4M2), 3) Great Keppel Island corals maintained at their site of origin (October 2008; 10KK1, 10KK2), Magnetic Island corals translocated to Great Keppel Island (October 2008; 10MK1, 10MK2), Magnetic Island corals maintained at their site of origin (collected in December 2008; 12MM1, 12MM2), Great Keppel Island corals translocated to Magnetic Island (collected in December 2008; 12KM1, 12KM2), and wild corals from Magnetic Island (Magnetic 1, Magnetic 2). The corals 10MK1 and 10MK2 bleached from cold water, and the corals 12KM1 and 12KM2 bleached in warm water.

Principal components analysis (PCA) identified 2 main groups of samples, and explained 63% of the variance in the bacterial profiles (Figure 3.2). The PCA demonstrates that Magnetic Island 'wild' corals cluster separately from all control and translocated corals attached to racks. Magnetic Island 'wild' bacterial profiles are

mostly composed of *Roseobacter* sp.-related sequences and *Spongiobacter* sp.-related sequences, whereas samples collected from the racks are mainly composed of *Vibrio* sp.-affiliated sequences and *Diaphorobacter* sp.-related sequences.

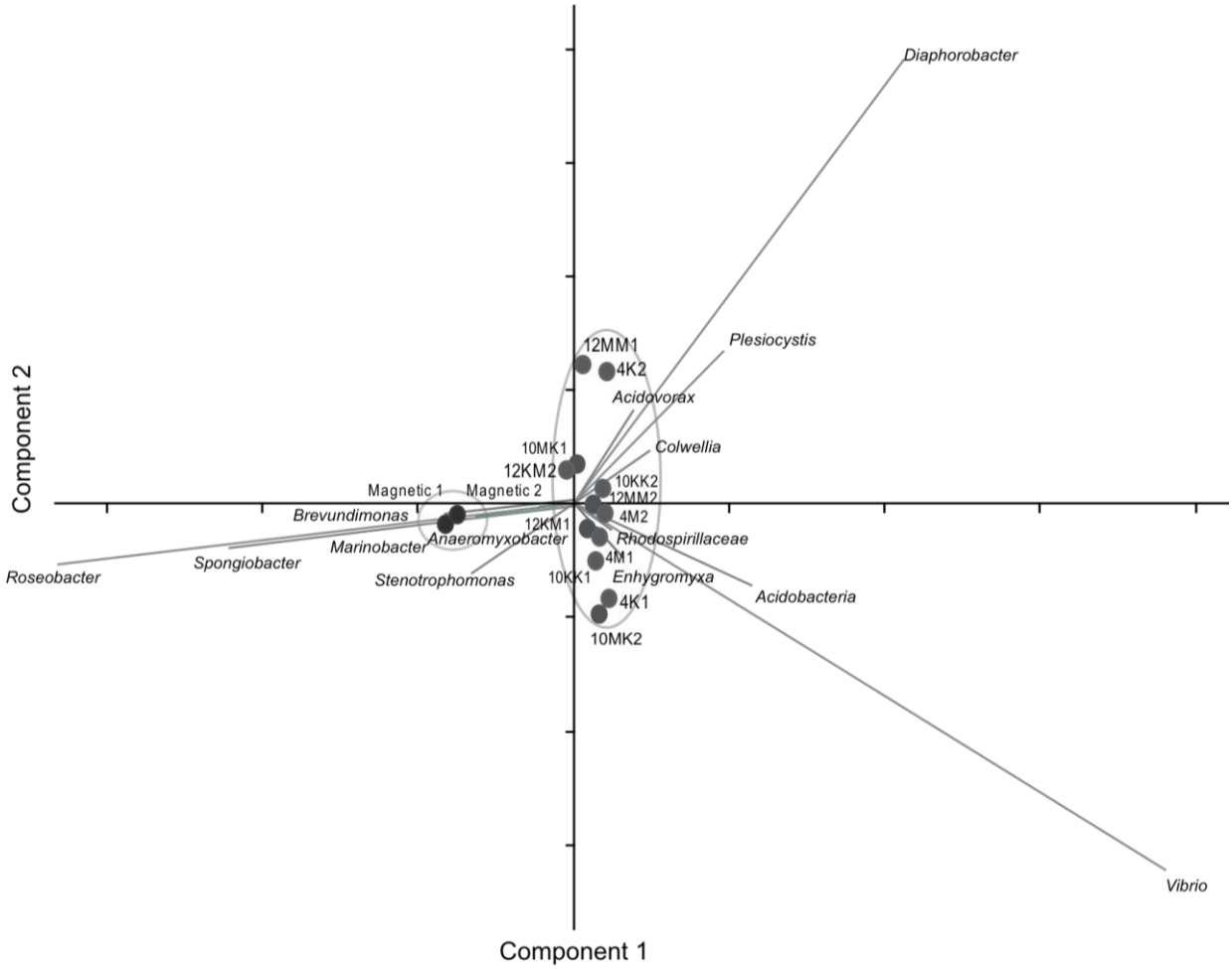

Figure 3.2 Principal components analysis of clone libraries comparing translocated coral colony bacterial communities with 'wild' Magnetic Island coral bacterial communities.

Comparison of DGGE profiles between experimental samples collected from racks and wild samples retrieved from Magnetic Island and Great Keppel Island also demonstrate that bacteria associated with corals on racks differ from those associated with wild corals. Regardless of the source reef, corals placed on racks have identical bacterial profiles (Figure 3.3), with most bands recovered corresponding to *Vibrio* sp.

(Table 3.1). These profiles, however, differed from Keppel Island and Magnetic Island coral-associated bacterial DGGE profiles. Some of the dominant bacteria recovered from Keppel Island 'wild' corals affiliated with *Flavobacterium* sp., *Achromobacter* sp. and *Bordetella* sp., whereas sequences retrieved from Magnetic island corals were related to *Roseobacter* sp., *Anaeromyxobacter* sp., *Marinobacter* sp., *Pseudomonas* sp., *Vibrio* sp., and *Spongiobacter* sp. (Table 3.1). The nMDS illustrates how each sample profile originating from the same location (Keppel Is, Magnetic Is or racks) clusters together in a group separate from other locations (Figure 3.4).

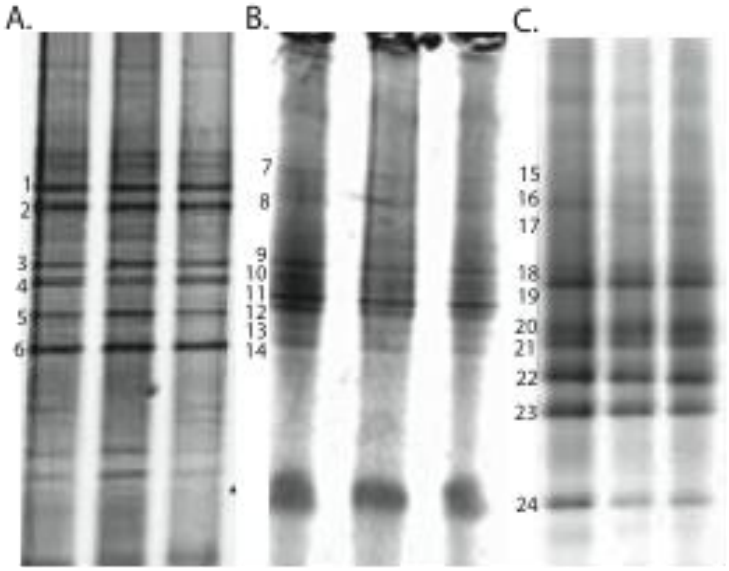

Figure 3.3 Denaturing gradient gel electrophoresis for samples collected from: A) control and translocated rack corals (4KK and 4MM), B) Great Keppel Island 'wild' samples, and C) Magnetic Island 'wild' samples. Numbers correspond to band stamps in Table 1.

Table 3.1 Affiliation of DGGE bacterial sequences retrieved from coral colonies from Magnetic Island (n=3) Keppel Island (n=3), and racks (n=6)

Band No.	Closest relative and database accession number	Alignment (bp)	Similarity* (%)	Taxonomic description
Rack samples				
1	*Vibrio parahaemolyticus* (GU272134)	303/306	99	*γ -Proteobacteria*
2	*Vibrio parahaemolyticus* (GU272135)	306/315	97	*γ -Proteobacteria*
3	*Vibrio alginolyticus* (GU272121)	303/306	99	*γ -Proteobacteria*
4	Uncultured bacterium (GU184532)	298/315	94	
5	*Vibrio parahaemolyticus* (GU272134)	306/315	97	*γ -Proteobacteria*
6	Uncultured bacterium (AY868600)	305/315	96	
Keppel Island				
7	Uncultured bacterium (AY771738)	301/308	97	*Bacteroidetes*
8	*Flavobacterium* sp. (EU000241)	296/299	98	*Bacteroidetes*
9	*Flavobacterium* sp. (AY771737)	287/290	98	*Bacteroidetes*
10	*Achromobacter* sp. (FN582313)	277/287	96	*β-Proteobacteria*
11	*Bordetella* sp. (AF511432)	277/287	96	*β-Proteobacteria*
12	*Achromobacter* sp. (EU304280)	296/299	98	*β-Proteobacteria*
13	Uncultured bacterium (AY136158)	287/290	98	*γ-Proteobacteria*
14	Uncultured bacterium (FJ205084)	298/315	94	
Magnetic Island				
15	*Roseobacter* sp. (DQ412059)	277/281	98	*α-Proteobacteria*
16	*Anaeromyxobacter* sp. (EU331403)	302/307	98	*δ-Proteobacteria*
17	*Marinobacter* sp. (EU624424)	303/308	98	*γ -Proteobacteria*
18	*Pseudomonas* sp. (EU770402)	303/309	99	*γ-Proteobacteria*
19	*Spongiobacter* sp. (AB205011)	297/312	95	*γ-Proteobacteria*
20	Uncultured bacterium (EU628068)	309/312	99	
21	*Vibrio* sp. (EU143360)	272/277	98	*γ-Proteobacteria*
22	*Pseudomonas* sp. (EU770402)	277/281	98	*γ-Proteobacteria*
23	*gamma proteobacterium* (DQ351795)	309/312	99	*γ-Proteobacteria*
24	*Spongiobacter* sp. (AB205011)	305/313	97	*γ-Proteobacteria*

*Sequences were aligned to the closest relative using BLAST (Altschul, 1997). The similarity was calculated with gaps not taken into account.

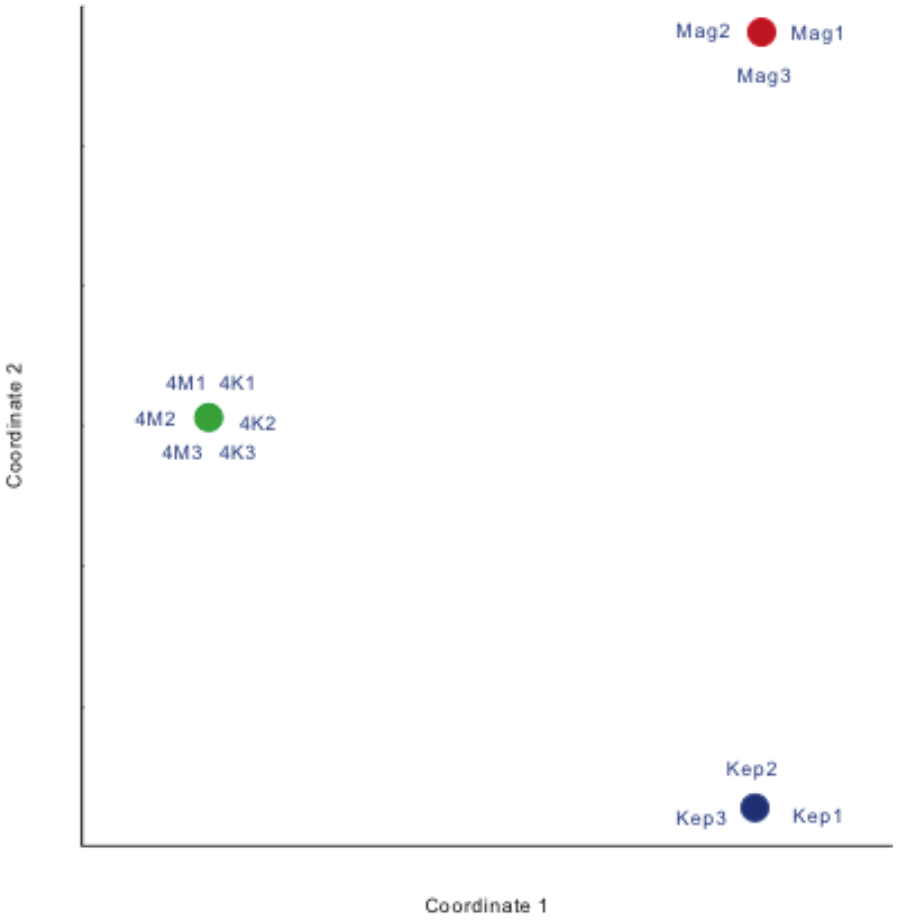

Figure 3.4 Non-metric multi-dimensional scaling of DGGE profiles for Magnetic Island wild corals (Mag), Great Keppel Island 'wild' corals (Kep), rack samples collected from Magnetic Island in April (4M), and rack samples collected from Great Keppel Island in April (4K).

3.3.2 Translocated corals experienced bleaching but no shifts in coral-associated bacteria

All translocated corals experienced bleaching due to temperature stress associated with the new environment in which they were placed. In October 2008, corals originating from Magnetic Island and translocated to Great Keppel Island (MK) experienced cold water bleaching, with temperatures averaging 22°C± .5°C (AIMS Data Centre). All ten MK corals displayed visual signs of paling, although all KK corals remained visually healthy. Three MK corals died and the rest recovered in the following months. Conversely, corals originating from Great Keppel Island and

translocated to Magnetic Island bleached due to high temperatures in December 2008, with temperatures averaging 30°C± .5°C (AIMS Data Centre). All 20 KM corals showed visual signs of bleaching, whereas the 10 MM corals remained healthy.

Comparisons of clone libraries for baseline (pre-translocation) samples at the two sites with those from both Great Keppel Island corals during the winter bleaching event and Magnetic Island corals during the summer bleaching event revealed that the same dominant bacteria were recovered from clone libraries, regardless of location or season. Thus, there was no evidence of shifts in bacterial communities due to temperature stress. The dominant bacteria retrieved from clone libraries affiliated with *Vibrio* sp. Other bacteria retrieved from most clone libraries affiliated with *Acidobacteria* sp., *Diaphorobacter* sp., *Plesiocystis* sp., *Colwellia* sp. and *Stenotrophomonas* sp. (Figure 3.1). According to the PCA analysis, *Vibrio* sp. and *Diaphorobacter* sp. were the most dominant members of the bacterial communities, as indicated by the prominent vectors (Figure 3.2). Samples from each time point did not cluster together, indicating that there was high variability between samples. Moreover, there were no distinguishable patterns in variation between clone libraries from control and translocated corals that could be detected in the PCA.

Five replicate samples from each treatment (MM, MK, KK and KM) were analysed by DGGE for each of 5 different months at both locations (data not shown). DGGE analysis demonstrated that bacterial profiles did not change throughout 2008 for any of the treatments. Figure 3.5 shows similar DGGE profiles for corals before transplantation, corals at Keppel Island during cold water bleaching and corals on Magnetic Island during warm water bleaching, confirming that bleaching did not change the bacterial community structure.

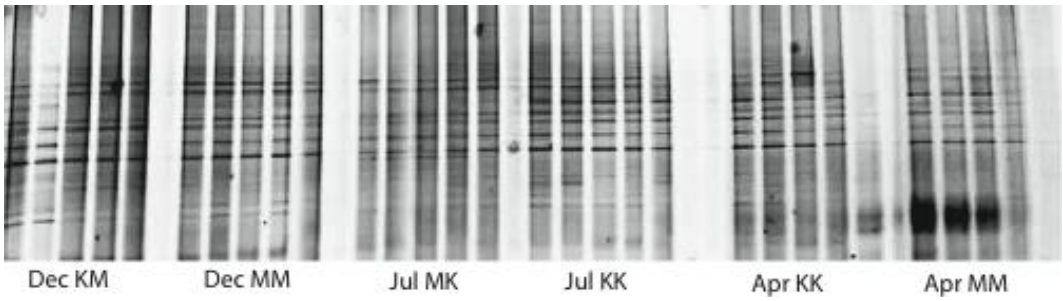

Figure 3.5 DGGE profiles for samples collected from: Magnetic Island during the warm water bleaching in December (Dec KM, Dec MM), Great Keppel Island during the cold water bleaching in July (Jul MK, Jul KK), and samples collected in April from both island locations before the transplantation (Apr KK, Apr MM).

3.4 Discussion

Clone library construction of samples from corals placed on racks revealed a different bacterial community from that found in a previous study examining 'wild' corals from Magnetic Island (see Chapter 2). PCA analysis of clone libraries demonstrates that the bacterial communities found on 'wild' corals differ dramatically from those collected from racks located on Magnetic Island. This was further confirmed by DGGE, which shows that bacteria associated with the 'wild' corals near both Magnetic Island and Great Keppel Island differed from those found on the corals placed on racks (Figure 3.3 and 3.4). Interestingly, the dominant bacterial sequences of rack corals were the same, regardless of whether the corals were from Magnetic Island or Great Keppel Island. Conversely, DGGE analysis shows that 'wild' Great Keppel samples had different bacterial profiles than those recovered from 'wild' Magnetic Island profiles. It is possible that the process of placing both control and translocated corals on metal racks to facilitate attachment to the reef, may have affected the bacteria present and resulted in the same bacterial sequences appearing on all corals placed on racks. The presence of the same bacterial sequences on both control and translocated corals, regardless of translocation to a different environment, suggests that the racks

played a greater role in structuring the bacterial community than the environment to which they were translocated.

It is possible that by-products or nutrients derived from the galvanized iron racks may have induced shifts in the bacterial communities on the corals. The dominant bacteria found on all rack corals (*Vibrio* sp.) are capable of scavenging for iron (Simpson and Oliver 1987). Moreover, the second most abundant bacteria, *Diaphorobacter* sp. is capable of nitrate-dependent iron oxidation (Taft 2009). Iron availability is a major limiting factor for the growth of certain bacteria (Guerinot 1994, Ratledge and Dover 2000) and it is likely the metal racks provided ample sources of this nutrient that normally occurs in trace amounts. The bacteria capable of producing high levels of iron-binding agents, such as siderophores, would be able to flourish under such conditions, which may explain the presence of these bacteria on rack corals. It has been hypothesized that bacteria play a key role in degradation of limiting nutrients that may serve as food for corals, and as nutrient sources change, the relative amounts of degrading bacteria associated with corals can change (Reshef et al. 2006). Klaus et al. (2007) has shown that bacterial communities of *Diploria strigosa* differed with proximity to coastal pollution, indicating that nutrient inputs can affect bacteria associated with corals. Therefore, it is likely that a change in iron availability could have altered the relative amounts of *Vibrio* sp. and *Diaphorobacter* sp. hosted by the coral.

Another possibility is that all the samples were somehow contaminated during DNA extraction. However, this explanation is unlikely given that PCR amplification, as well as downstream analyses such as clone library construction and DGGE, were extremely difficult and took multiple tries involving several additional DNA purification steps. Collaborators encountered similar problems while using these

samples for gene expression analysis (L. Bay, pers. comm.). Although RNA yields were high, RNA would not hybridize to microarray chips. It is possible that corals placed on racks contained a different chemical composition that inhibited the molecular methods used in this experiment. If samples had merely been contaminated, PCR amplification would have been far easier. Furthermore, DNA was extracted three different times using new reagents to eliminate the possibility of lab contamination, and the same result was reached. Another possibility is that the samples were somehow contaminated before lab analysis, for example they may have been contaminated in the field. However, the samples were collected throughout the course of a year using different liquid nitrogen storage dewars, plastic bags, and liquid nitrogen; it is highly unlikely that all samples were contaminated with the same bacteria during every collection.

The rack coral bacterial community was maintained for at least one year, i.e. throughout the experiment, however the community may have persisted for longer, as corals were left on racks for 3 years prior to experimentation. Thus, it appears that this bacterial community was stable. Furthermore, the bacterial communities did not shift through two bleaching events, i.e. neither following cold water bleaching on Great Keppel Island nor warm water bleaching on Magnetic Island. These results are contrary to previous findings, which have demonstrated changes in bacterial communities as a result of coral bleaching (Bourne et al. 2008). However, Bourne et al. (2008) found that bleaching corresponded to proliferation of *Vibrio* sp., which were already the dominant group on rack corals, indicating that bacterial communities had already been altered due to an unknown environmental factor. The increase in *Vibrio* sp. in bleached corals was hypothesized to be due to secondary infection during bleaching (Bourne et al 2008), however, the stable nature of the association found in

this study for over one year implies that the relationship with *Vibrio* sp. may have been mutualistic or at least neutral.

In this study, it appears that the metal racks on which the corals were placed were more important in shaping bacterial communities associated with *Acropora millepora* than the reef locations to which they were translocated. Nutrient inputs, particularly metal-derived Fe2+, may influence the bacteria present on corals and should be considered in manipulative studies that rely on maintanence of healthy colonies. This is a particularly important consideration for studies that use iron nails to mark the location of corals. in addition, changes in bacterial communities on corals in the vicinity of shipwrecks may have larger implications for coral diversity. For instance, Barott et al. 2010 noted alterations in the benthic community of Millenium atoll in the Republic of Kiribati as a consequence of iron leaching from a shipwreck's corroding hull. Thus, iron leaching may have negative impacts on coral health through changes in their microbial communities and should be investigated further.

Chapter 4.0 Bacterial communities of juvenile corals infected with different Symbiodinium (dinoflagellate) clades

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Littman, R. A., Willis, B. L. and Bourne, D. G. (2009) Bacterial communities of juvenile corals infected with different *Symbiodinium* (dinoflagellate) clades. *Marine Ecology Progress Series*, **389**: 45-59.

All the data was collected and analyzed by R. Littman, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.

4.1 Introduction

For decades, research on coral symbioses has focused on *Symbiodinium* and its photosynthesis, revealing the important roles that the algal endosymbiont plays in providing a source of carbon necessary for coral growth and in assisting calcification (Barnes & Chalker 1990; Muller Parker & D'Elia 1997). In contrast, the roles of bacterial and archaeal associates that populate the mucus and tissue layers of corals are poorly understood. Potential benefits for the coral holobiont (which includes the host and all microbial partners) can be inferred from bacterial isolates and affiliated sequences retrieved from previous studies investigating coral-associated bacteria. Some coral-associated bacteria are known to fix nitrogen and carbon and may pass products to the coral host (Williams et al. 1987; Shashar et al. 1994; Cooney et al. 2002; Rohwer et al. 2002; Lesser et al. 2004). Other bacteria have been found to produce secondary metabolites such as antibiotics (Castillo et al., 2001). Moreover, bacteria isolated from the mucus of healthy *Acropora palmata* have been shown to inhibit growth of potentially pathogenic microbes (Ritchie 2006). Thus, bacterial communities may play important roles in maintaining coral health.

Recently, the role and diversity of algal and bacterial symbionts, as well as the possibility that corals may adapt to environmental conditions by altering their symbiont communities (Buddemeir & Fautin 1993; Reshef et al. 2006) have been the focus of a number of studies. It has been shown that different genetic types of *Symbiodinium* can differentially affect the physiology of the coral host. For example, corals containing *Symbiodinium* clade D tend to be more tolerant to heat stress (Baker 2004, Berkelmans & van Oppen 2006), although species-specific coral-*Symbiodinium* interactions may modify this general pattern (Abrego et al. 2008). Furthermore, both *Acropora tenuis* and *A. millepora* juveniles grow 2-3 times faster when associating

with *Symbiodinium* clade C1 compared to those associated with clade D (Little et al. 2004). Faster growth of corals infected with *Symbiodinium* C1 may reflect a greater contribution of the symbiont to host nutrition through faster rates of population growth inside the host (Fitt 1985) and/or doubling of the rate of photosynthate translocation to host tissues (Cantin et al. 2009). However, to date, no studies have investigated how different symbiotic *Symbiodinium* clades within the coral host might affect other microbial partners in the coral holobiont. As a multispecies mutualism, the relationship between corals and their symbionts may have an additional dimension; *Symbiodinium* endosymbionts may interact differentially with bacterial associates competing for space within the host to shape aspects of holobiont physiology. For instance, most (as high as 98%) of the net carbon assimilated by *Symbiodinium* is released as exudates, much of it excreted into the mucus (Ikeda & Miyachi 1995). Mucus composition is potentially important in structuring microbial communities (Ritchie & Smith 2004). Ritchie and Smith (1997) demonstrated that selective carbon source utilization by cultured bacteria differs among 11 coral species. Variation in photosynthetic contributions by different *Symbiodinium* could therefore affect the composition of coral mucus, indirectly impacting the coral microbiota. Changes in algal symbionts associated with corals may lead to differences in the bacterial populations on corals and this may have larger implications in times of environmental stress, as changes in one component of the microbial community may cause a complete shift in coral-associated microbial communities.

To further our understanding of possible interactions between bacterial and *Symbiodinium* communities associated with coral, this study compared the bacterial community profiles of 9-month old juvenile colonies of the corals, *Acropora millepora* and *Acropora tenuis,* that had been experimentally infected with two different clades

of *Symbiodinium*, C1 and D. Juvenile corals were raised from larvae to enable manipulation of the *Symbiodinium* type harboured upon initiation of symbiosis. Bacterial profiles of juvenile corals were compared to those of adult corals containing the same clade of *Symbiodinium* to determine if age affects the composition of bacterial communities. A second sample of *A. tenuis* juveniles (12-month old) containing C1 and D collected three months later in summer was analysed to determine whether temporal changes, such as increase in temperature, might differentially influence bacterial communities associated with corals harbouring different *Symbiodinium* types. Three culture-independent profiling methods were used to analyse bacterial associates, denaturing gradient gel electrophoresis (DGGE), clone library construction, and terminal restriction fragment length polymorphism (tRFLP) to cross-validate findings.

4.2 Materials and methods

4.2.1 *Gamete collection, larval settlement and* Symbiodinium *infection*

Juveniles of the corals, *Acropora tenuis* and *A. millepora*, were produced and experimentally infected with *Symbiodinium* C1 or D as described in Abrego et al. (2008). In summary, adult colonies of both species were collected from the upper reef slope in Nelly Bay, Magnetic Island (19º10's 146º50'E) just prior to spawning in November 2006 and placed in separate tanks onshore. Following spawning, gametes were collected from 4 colonies of each species and mixed in aquaria containing 1 micron filtered seawater to effect fertilization. Once cleavage was detected, embryos were moved into larger culture tanks supplied with flow-through 1 micron filtered seawater throughout larval development. When larvae commenced searching for settlement sites, sterilized terracotta tiles were placed on the bottom of culture tanks to

provide settlement surfaces. Autoclaved coralline algae was then added to provide settlement cues for larvae. Following settlement, tiles were divided into separate tanks for infection with algal endosymbionts.

Settled *A. tenuis* and *A. millepora* corals were experimentally infected with both homologous and heterologous *Symbiodinium* clades. Algal isolates were prepared by airbrushing adult colonies of 1) *Acropora tenuis* to collect *Symbiodinium* C1, and 2) *A. millepora* to collect *Symbiodinium* D. Colonies were collected from Magnetic Island, where these species are known to be associated with the targeted *Symbiodinium* clades (Little et al. 2004). Tissue slurries were centrifuged (3500 rpm, 3 minutes) to pellet *Symbiodinium* cells and liquid was removed. Pellets were re-suspended in freshly filtered seawater and repeatedly washed until coral tissue was removed. Algal cells were counted under an Olympus BH-2 light microscope (Olympus Corp, Shinjuku-ku, Tokyo) using a haemocytometer to determine concentrations of cells. Isolates were diluted to approximately the same density of *Symbiodinium* cells (about 400 cells per ml) and added in equal amounts into tanks containing settled larvae.

Following infection with the appropriate algal symbiont, colonies were sampled and *Symbiodinium* clade was verified by single stranded conformation polymorphism (SSCP). At the approximate size of 3-5 polyps, juvenile corals were returned to the parental habitat, where they remained until sampled. Tiles containing juvenile corals were placed on steel rods and suspended horizontally between pairs of metal star-pickets on the reef flat in Nelly Bay, Magnetic Island.

4.2.2 Sample collection and processing

Nine months (September 2007) and twelve months (December 2007) after coral juveniles were out-planted to the reef, six juveniles containing *Symbiodinium* C1 and

six containing *Symbiodinium* D from each of *Acropora tenuis* and *A. millepora* (i.e. 24 juveniles in total at each sample time) were removed from the settlement tiles by detaching colonies with a sterile scalpel (Table 4.1). Water temperatures were 25°C in September and had reached 30°C in December. Whole juvenile colonies were rinsed with artificial sea water, placed in cryovials and stored at -80ºC. The number of surviving juveniles was counted and the size of each colony measured at each sample time.

4.2.3 DNA extraction and purification

DNA was extracted by suspending an entire juvenile colony in 0.5 ml of buffer (0.75M Sucrose, 40mM EDTA, 50mM Tris, pH 8.3) and following the extraction protocol outlined previously (Bourne et al. 2008). The DNA pellet was suspended in 30 µl of sterile milli-Q water and the total volume was loaded on a 1.2% low-melting agarose gel. DNA was purified by using electrophoresis and cutting high quality DNA (>2kb) from the gel. The agarose was then removed from the sample by using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA was recovered from the Qiagen column with two 30 µl washes of sterile milli-Q water.

4.2.4 PCR amplification of 16S ribosomal RNA gene

Universal primers 63f and 1387r (Marchesi et al. 1998) were used to amplify the 16S rRNA genes from extracted DNA for bacterial clone library construction. Amplification of the 16S rRNA genes for terminal restriction length fragment polymorphism analysis was performed using the D4 labelled 63F primer and 1389R primer. The PCR mixtures were prepared as previously described in Chapter 2.2.3. PCR was performed with Applied Biosystems 2720 thermocycler and programmed

with an initial 3 min step at 94ºC and thirty-five cycles consisting of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1.5 minutes and a final extension for 10 minutes at 72ºC.

For denaturing gel electrophoresis, the bacterial 16S rRNA gene was amplified using primers 1055f and 1392R-GC (Ferris et al. 1996). PCR reactions were prepared as stated in section 2.2.3. DGGE PCR reactions were carried out using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) thermocycler. Temperature cycling was performed using a touchdown protocol (Ferris et al. 1996).

4.2.5 Single stranded conformation polymorphism (SSCP)

SSCP was used to identify the *Symbiodinium* type present in each juvenile at each sampling to ensure that corals still harboured the same C1 or D *Symbiodinium* type with which they were initially infected. The 18S internal transcribed spacer 1 (ITS1) region of *Symbiodinium* clades was amplified using ITSF and ITSR primers (van Oppen et al. 2001). PCR products were diluted 1:3 with formamide dye and denatured by placing them at 95°C for 5 minutes and then immediately on ice. Products were loaded on 4% acrylamide gel and run on a Gel-Scan 3000 (Corbett Robotics, Sydney, Australia) with 0.5X TBE buffer (0.01M Tris base, 0.01M boric acid and 0.2mM Na_2 EDTA; pH adjusted to 8.3). Standards for ITS 1 type C1, C2 and D were also loaded in every fifth well for comparison. Temperature was set at 22°C and run at 1200v for 25 minutes. Gels were imaged using Gel-Scan 3000 software (Corbett Robotics, Sydney, Australia).

4.2.6 Clone library construction

The amplified bacterial DNA was ligated into TOPO®-cloning vector following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Ligations were submitted to the Australian Genome Research Facility for transformation, cloning and subsequent sequencing. Two replicate libraries of 96 clones each were sequenced for samples containing each *Symbiodinium* clade for both *Acropora* species. Dominant bacterial 16S rRNA gene sequences retrieved from each clone library were analysed using principal components analysis (PCA) to determine which sequences (affiliated at the genera level) were important in driving differences between the libraries. Due to the high amount of variability in each library, only 16S rRNA gene sequences which constituted 2% or more of any clone library were included in the PCA analysis.

4.2.7 Denaturing gradient gel electrophoresis analysis (DGGE)

Bacterial profiling was carried out using an INGENY phorU-2 (Ingeny International BV, Netherlands) DGGE system. PCR products were separated on gels as described in section 2.2.5. Clear bands were excised from the gel and placed in 100 µl of sterile milli-Q water to elute DNA from the acrylamide gel. The DNA bands were re-amplified and run on the DGGE gel to ensure correct migration and purity of the product. Products that showed one distinct band with the correct mobility on the DGGE were directly sequenced. Using the sequences recovered from cut bands, a presence/absence matrix was constructed for the DGGE fingerprints and analyzed by non-metric multi-dimensional scaling (nMDS) using Euclidian distances.

4.2.8 Terminal restriction fragment length polymorphism analysis (tRFLP)

Prior to restriction digestion, 3 replicate PCR products for each sample were pooled and purified using the QIAquik PCR Purification Kit (Qiagen, Hilden, Germany). The restriction reaction was carried out as described in section 2.2.6.

Digested samples were separated on a Beckman Coulter CEQ 8800 sequencer (Fullerton, CA, USA). Fragment analysis was performed on an 8 capillary array in fragment analysis mode using Beckman Coulter CEQ 8000 software (Fullerton, CA, USA). The parameters are outlined in section 2.2.6. Peak size and retention times were exported into T-align (Smith et al. 2006) and consensus peaks were determined by aligning replicate peak profiles and including any peaks within a range of 0.5 peak area. Peak profiles were then converted into a presence/absence matrix and visualized using non-metric multi-dimensional scaling (nMDS) with Euclidean distances.

4.2.9 Sequence phylogenetic analysis and diversity indices

Sequences from clone libraries were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al. 1996). Sequence data (approximately 700bp each) were aligned to the closest relative using the BLAST database algorithm (Altschul et al. 1997) and all sequence affiliations were determined by >97% identity to bacterial 16S rRNA gene sequences in the GenBank database. Sequence affiliations were cross-validated with the Greengenes database (http://greengenes.lbl.gov) (DeSantis et al., 2006) using the Hugenholtz and Ribosomal Database Project (RDP) (Maidak et al. 1996) alignments, and sequences that closely affiliated with uncultured bacteria were assigned taxonomic affiliations using RDP database alignments. Sequences were assigned operational taxonomic units (OTUs) by comparing sequences with the same affiliations and grouping those with >97% similarity. The OTUs of library clone and DGGE sequences were imported into the ARB software package (http://www.arb-home.de) (Ludwig et al., 2004), aligned against the Greengenes database (http://greengenes.lbl.gov) (DeSantis et al., 2006) that is compatible with ARB, followed by a manual correction of the alignment when necessary. Operational taxonomic units that were >2% of any juvenile clone library

were included in a phylogenetic tree with dominant bacterial sequences retrieved from adult samples (Littman et al. 2009) to determine which conserved adult bacteria are present on juvenile samples. DGGE sequences were also included in the tree to determine sequence affiliations as well as cross-validate sequence alignments with clone library data. Tree topologies were evaluated by reconstructing phylogenies using evolutionary distance (Phylip Distance Method with Jukes and Cantor model) analysis of aligned near full-length sequences (>1000 bp) (Ludwig et al. 1998). Regions of ambiguous sequence were removed from the analysis. Aligned, partial 16S rRNA gene sequences (<1000 bp) were subsequently inserted without changing the overall tree topology using the parsimony tool available within ARB. The nucleotide sequence data of clones and DGGE sequences appear in the GenBank nucleotide database under the accession numbers GQ301209-GQ301527.

Diversity indices were compared for adult and juvenile *Acropora millepora* and *tenuis* containing the same *Symbiodinium* clades. The Shannon-Weaver diversity index (Shannon & Weaver 1963), the Chao1 richness estimator (Chao 1987) and Fisher's Alpha log series richness index (Fisher et al. 1943) were calculated using the freeware program EstimateS Win 7.52 (Colwell 2006). Coverage values were calculated by the equation: $C = 1 - (n/N) \times 100$ where n is the number of unique clones and N the total number of clones examined in the libraries (Good, 1953). The diversity of clone libraries was further investigated by rarefaction analysis (Hurlbert 1971; Heck et al. 1975; Simberloff 1978). Rarefaction curves were produced by using the analytical approximation algorithm of Hurlbert (1971). Calculations were performed with the freeware program aRarefact Win (Holland 1988).

4.3 Results

4.3.1 Bacterial community profiles of 9-month old juveniles

A total of 81 *Acropora tenuis* juveniles from the *Symbiodinium* D treatment and 126 from the *Symbiodinium* C1 treatment were alive when surveyed at 9 months (September 2007). On average, D juveniles contained 23 ± 4 polyps (0.5-1 cm in diameter), in contrast to C1 juveniles, which contained approximately 51 ± 9 polyps and were visibly larger (1-2 cm). For *A. millepora*, 63 juveniles from the D treatment and 123 juveniles from the C1 treatment were alive at the time of the September sample. Average polyp counts (D-juveniles: 18 ± 5; C1 juveniles: 50 ± 8) and colony sizes (D-juveniles: 0.5-1.0 cm; C1-juveniles: 1-2 cm) of the *A. millepora* juveniles were similar to those of *A. tenuis* juveniles from the respective *Symbiodinium* C1 and D treatments (Table 4.1).

Table 4.1 Sampling design and survey results

Age	9 months				12 months		Adult*	
Species	*A. tenuis*		*A. millepora*		*A. tenuis*		*A. tenuis*	*A. millepora*
***Symbiodinium* clade**	C1	D	C1	D	C1	D	C1	D
Replicate	6	6	6	6	6	6	3	3
Size (cm)	1-2	0.5-1	1-2	0.5-1	2-3	1	NA	NA
Number alive	126	81	123	63	112	18	NA	NA

*Sample data from Littman et al. 2009

Bacterial communities identified within 16S rRNA gene clone libraries of all 9-month old coral juveniles were dominated by *α*- and *γ-Proteobacteria* affiliated sequences. No differences in dominant phylogenetic groups at the class level were discernable among corals containing different *Symbiodinium* clades (Appendix 2).

Retrieved sequence diversity within the libraries examined at the genus and family level demonstrated high bacterial diversity, and there were few similarities between coral juveniles, either when compared between *Acropora tenuis* and *A. millepora* juveniles associated with the same *Symbiodinium* type, or when compared between C1 and D juveniles within a species. Each juvenile clone library correlated with different dominant bacteria genera, and also there were few similarities between any of the bacterial communities associated with the replicate juveniles within each coral species (Table 4.2). However, a few bacterial sequence OTUs appeared within replicate libraries more frequently suggesting that juvenile libraries may have limited structure. The sequences that were recovered from multiple libraries, from both *A. millepora* and *A. tenuis* libraries hosting both *Symbiodinium* clades C1 and D, include OTU-007 (affiliated with *Flavobacteria* sp.), OTU-009 (related to *Muricauda* sp.), OTU-168 (related to *Roseobacter* sp.), OTU-290 (related to *Achromobacter* sp.), OTU-308 (related to *Pseudomonas* sp.) and OTU-310 (affiliated to *Serratia* sp.). Two OTUs (148 and 149) related to *Brevundimonas* sp. appeared within most libraries while a *Stenotrophomonas* sp.-related sequence (OTU-314) represented a large component of each library (between 2.2 – 31.7% of clones). Nevertheless, most common sequences listed above do not appear in every library and vary drastically in their relative proportions (Table 4.2). In addition, all juvenile clone libraries possessed a large number of independent bacterial ribotypes that only appeared once in each library, which is represented by the large number of OTU groups identified (Appendix 2). Due to the high variability and diversity within all juvenile libraries, no consistent patterns could be detected between samples harbouring clade C1 or D *Symbiodinium* by directly comparing relative abundances of retrieved 16S rRNA gene sequences.

Table 4.2 Proportions of dominant Operational Taxonomic Units retrieved from 16S rRNA gene juvenile clone libraries (%). Sequences included comprise >2% of any juvenile clone library. *Sequences were aligned to the closest relative using BLAST (Altschul, 1997). The similarity was calculated with gaps not taken into account. MC: *A. millepora* hosting clade C1; MD: *A. millepora* hosting clade D; TC: *A. tenuis* hosting clade C1; TD: *A. tenuis* hosting clade D; S-TC: *A. tenuis* hosting clade C1 sampled in summer; S-TD: *A. tenuis* hosting clade C1 sampled in summer

Clone	Closest Relative*	Identity*	Affiliation	MC1	MC2	MD1	MD2	TC1	TC2	TD1	TD2	S-TC1	S-TC2	S-TD1	S-TD2
OTU-001	*Acidobacteria* (DQ289940)	98	*Acidobacteria*						2.4	1.2					
OTU-004	*Bacteroidetes* bacterium (AY162097)	99	*Bacteroidetes*			1.2	1.1	2.4	3.5						
OTU-007	*Flavobacteria* (AF277542)	98	*Bacteroidetes*	4.3		1.2	2.3	1.2	1.2	1.2		8.3	5.3		
OTU-009	*Muricauda* sp. (AY576744)	99	*Bacteroidetes*	2.2		1.2		2.4				2.1	2.6		
OTU-015	*Lyngbya* sp. (AB045906)	93	*Cyanobacteria*	3.2											
OTU-016	*Oscillatoria* sp. (AB058224)	96	*Cyanobacteria*	1.1		2.4	2.3								
OTU-020	*Synechococcus* sp. (AF132772)	91	*Cyanobacteria*	4.3											
OTU-023	Unc bacterium (AF424415)	95	*Firmicutes*	1.1		2.4									
OTU-024	Unc bacterium (AY258098)	97	*α-proteobacteria*	1.1		2.4									
OTU-058	Unc bacterium (AY942776)	97	*α-proteobacteria*			2.4	5.7								
OTU-064	Unc bacterium (AY568808)	95	*δ-proteobacteria*				4.5								
OTU-011	Unc bacterium (EF378470)	97	*Bacteroidetes*					1.2				6.3	1.3		
OTU-144	*Agrobacterium* sp. (AB247617)	99	*α-proteobacteria*	2.2			1.1			1.2					
OTU-148	*Brevundimonas* sp. (AB426561)	99	*α-proteobacteria*				1.1	13.4	1.2	25.9	21.1	10.4	2.6	1.4	3.1
OTU149	*Caulobacteraceae* (DQ857204)	99	*α-proteobacteria*	2.2	2.3				4.7		7.8				
OTU-150	*Erythrobacter* sp. (DQ985055)	98	*α-proteobacteria*		4.7	2.4									3.1
OTU-153	*Kordiimonas* sp. (AY682384)	93	*α-proteobacteria*	1.1					2.4						
OTU-155	*Mesorhizobium* sp. (DQ917826)	98	*α-proteobacteria*				3.4								6.3
OTU-161	*Rhodobacteraceae* (AY962292)	99	*α-proteobacteria*	4.3					1.2						
OTU-165	*Rhodopseudomonas* sp. (AY428572)	93	*α-proteobacteria*				4.5								
OTU-168	*Roseobacter* sp. (AY745856)	99	*α-proteobacteria*					1.2	1.2	3.5	3.3		5.3	4.2	
OTU-170	*Ruegeria* sp. (AJ391197)	99	*α-proteobacteria*				3.4		1.2					6.9	
OTU-171	*Silicibacter* sp. (AY369990)	99	*α-proteobacteria*	1.1			3.4							1.4	
OTU-173	*Sphingomonas* sp. (EU817494)	99	*α-proteobacteria*									8.3	2.6		3.1
OTU-177	Unc *α-proteobacterium* (AY499896)	95	*α-proteobacteria*	6.5			1.1								
OTU-184	Unc *α-proteobacterium* (AJ890099)	99	*α-proteobacteria*	3.2		1.2									
OTU-191	Unc *α-proteobacterium* (AJ633940)	99	*α-proteobacteria*			7.2									
OTU-202	Unc *α-proteobacterium* (AM162572)	89	*α-proteobacteria*				3.4								
OTU-218	Unc *α-proteobacterium* (DQ107390)	99	*α-proteobacteria*					1.2	2.4						
OTU-262	Unc *α-proteobacterium* (DQ003179)	99	*α-proteobacteria*							3.5					
OTU-279	Mucus bacterium (AY654746)	99	*α-proteobacteria*	5.4		4.8									
OTU-290	*Achromobacter* sp. (EU275167)	100	*β-proteobacteria*		7.0	1.2				8.2	2.2				
OTU-291	*Delftia* sp. (EU019989)	99	*β-proteobacteria*							3.5					
OTU-294	Unc *β-proteobacterium* (EF061949)	99	*β-proteobacteria*			3.6									
OTU-293	Unc *β-proteobacterium* (AB288554)	99	*β-proteobacteria*	1.1						3.5					
OTU-299	*Alteromonas* sp. (EU529840)	100	*γ-proteobacteria*	3.2								2.1		1.4	
OTU-300	*Escherichia* sp. (AM087500)	99	*γ-proteobacteria*			1.2					1.1	4.2			3.1
OTU-308	*Pseudomonas* sp. (AY014801)	99	*γ-proteobacteria*			3.6	3.4	1.2		1.2		2.1		6.9	
OTU-310	*Serratia* sp. (AY566180)	99	*γ-proteobacteria*			1.2	3.4			1.2	2.2				
OTU-313	*Spongiobacter* sp. (AB205011)	98	*γ-proteobacteria*			1.2		2.4		1.2	1.1				
OTU-314	*Stenotrophomonas* sp. (AM402950)	100	*γ-proteobacteria*	2.2	27.9	8.4	4.5	31.7	8.2	29.4	24.4			2.8	
OTU-315	*Thalassomonas* sp. (AY194066)	98	*γ-proteobacteria*	9.7			1.1		1.2						
OTU-348	*Vibrio* sp. (AJ316167)	99	*γ-proteobacteria*											13.9	
OTU-349	Unc *α-proteobacterium* (DQ146982)	99	*γ-proteobacteria*	5.4			1.1				1.1				
OTU-350	*Vibrio* sp. (EU372927)	99	*α-proteobacteria*												18.8

OTU-355	*Haliangium* sp. (AB062751)	97	*δ-proteobacteria*	3.6	
OTU-356	*Myxobacterium* (AB016469)	97	*δ-proteobacteria*	1.2	2.4

Bacterial community profiles assessed by denaturant gradient gel electrophoresis (DGGE) displayed variable and complex banding patterns for replicate samples of both coral species. Sequences recovered from replicate DGGE sample profiles affiliated with *Brevundimonas* sp., *Stenotrophomonas* sp., *Muricauda* sp., *Achromobacter* sp. and *Ruegeria* sp. and corresponded to sequences retrieved from many *Acropora tenuis* and *A. millepora* clone library sequences (Table 4.2). This confirms that some bacterial ribotypes were consistent despite high diversity and variability in bacterial profiles. The nMDS plots representative of DGGE profiles for colonies of both *A. tenuis* (Figure 4.1a) and *A. millepora* (Figure 4.1b) show limited grouping of samples containing each of the two *Symbiodinium* clades, indicating that, as suggested by the clone library data, there was no obvious relationship between clade of *Symbiodinium* harboured and bacterial community composition in juveniles of these two corals. Bacterial diversity fingerprints generated from tRFLP peak patterns were consistent with both clone library and DGGE analysis in showing no strong grouping or consistency in peak patterns for replicate juvenile samples (Figures 4.1c, 4.1d). While nMDS representation of tRFLP peak patterns displayed some grouping of *A. tenuis* juveniles containing clade D *Symbiodinium* (Figure 4.1c), other replicate samples were dispersed. C1 juveniles, in particular, displayed little consistency in this species. *A. millepora* juveniles (Figure 4.1d) differed between replicate samples possessing the same clade, for those associated both with *Symbiodinium* C1 and D (Figure 4.1d), again indicating no clear relationship between *Symbiodinium* type harboured and bacterial community composition.

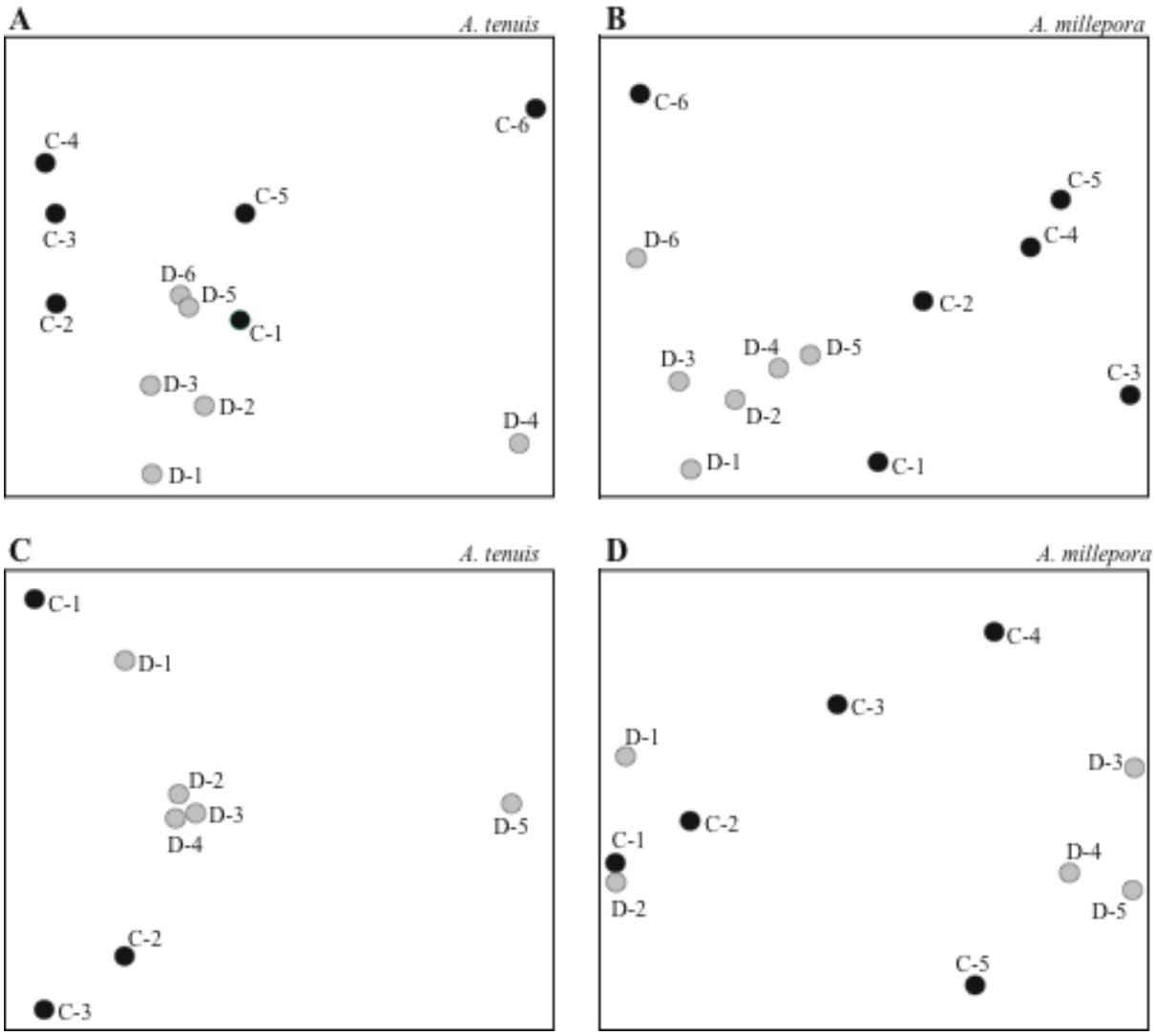

Figure 4.1 Comparison of 9-month juvenile corals containing clade C1 and D *Symbiodinium* by non-metric multidimensional scaling (nMDS) of bacterial profiles. A.) DGGE profiles of *A. tenuis*. B.) DGGE profiles of *A. millepora*. C.) t-RFLP profiles of *A. tenuis*. D.) t-RFLP profiles of *A. millepora*. C and D denote *Symbiodinium* clade present. Several replicate samples are missing in the t-RFLP analysis such as D-6, C-4, C-5 and C-6 for *A. tenuis* and C-6 for *A. millepora* due to poor reads by the sequencer and resulting loss of DNA after repeated trials which may contribute to the lack of pattern observed in the plot.

4.3.2 9-month juvenile vs. adult bacterial communities

Diversity parameters associated with clone libraries generated from the 9-month old juvenile samples were compared with bacterial diversities of adult corals associated with the corresponding *Symbiodinium* clades and collected from the same sites (Littman et al. 2009). Only 9-month clone libraries of the corals with the same

Symbiodinium clade to those of the adult libraries were included in the analysis, such as *Acropora millepora* harbouring clade D and *A. tenuis* hosting clade C1, to eliminate symbiont type as a possible influence. A total of 141 OTUs (operational taxonomic units grouped at 97% sequence identity) were identified within *Acropora millepora* juvenile coral libraries compared to only 53 OTUs from libraries of adult corals (Table 4.3). For both juvenile and adult *A. millepora* samples, libraries were pooled for the analysis, resulting in a total of 187 clones included in each analysis. For *A. tenuis*, 107 OTUs were identified within pooled juvenile libraries compared to 49 OTUs for adult *A. tenuis* (out of 179 and 183 clones respectively). Many sequences retrieved from juveniles affiliated with previously unclassified sequences and were the sole ribotypes within a defined OTU. Other bacterial ribotype richness (Chao1 and the Fisher abundance model) and evenness (Shannon-Weaver index) indices supported the conclusion that diversity was higher within juvenile samples (Table 4.3). The obtained sequences covered a high percentage of the diversity in the adult libraries (72% and 73% for *A. millepora* and *A. tenuis* libraries) in comparison to juvenile libraries (25% and 43% for *A. millepora* and *A. tenuis* libaries). Rarefaction curves confirmed the coverage calculations, with juvenile libraries failing to reach an asymptope in comparison to curves for adult corals (Figure 4.2). Although greater numbers of sampled clones would better represent bacterial diversity for all libraries, it was apparent that bacterial diversity of juvenile corals was vastly undersampled compared to adult corals.

Table 4.3 Diversity indices calculated from Operational Taxonomic Units (97% similarity) of 16S rRNA clone libraries.

Parameters	**Clone Library**			
	Adult		**Juvenile**	
	A. millepora	***A. tenuis***	***A. millepora***	***A. tenuis***
No. of clones analysed	187	183	187	179
Coverage of clone libraries (%)	71.7	73.2	24.6	43
Observed No. of OTUs	53	49	141	102
Shannon-Weaver diversity (H')	3.4	3.3	4.8	4.2
Chao1	80.2	72.2	474.2	280.3
Fisher's alpha (a)	24.7	21.9	260.9	98.4
Simpson's evenness (D)	21.74	19.7	238.2	50.1

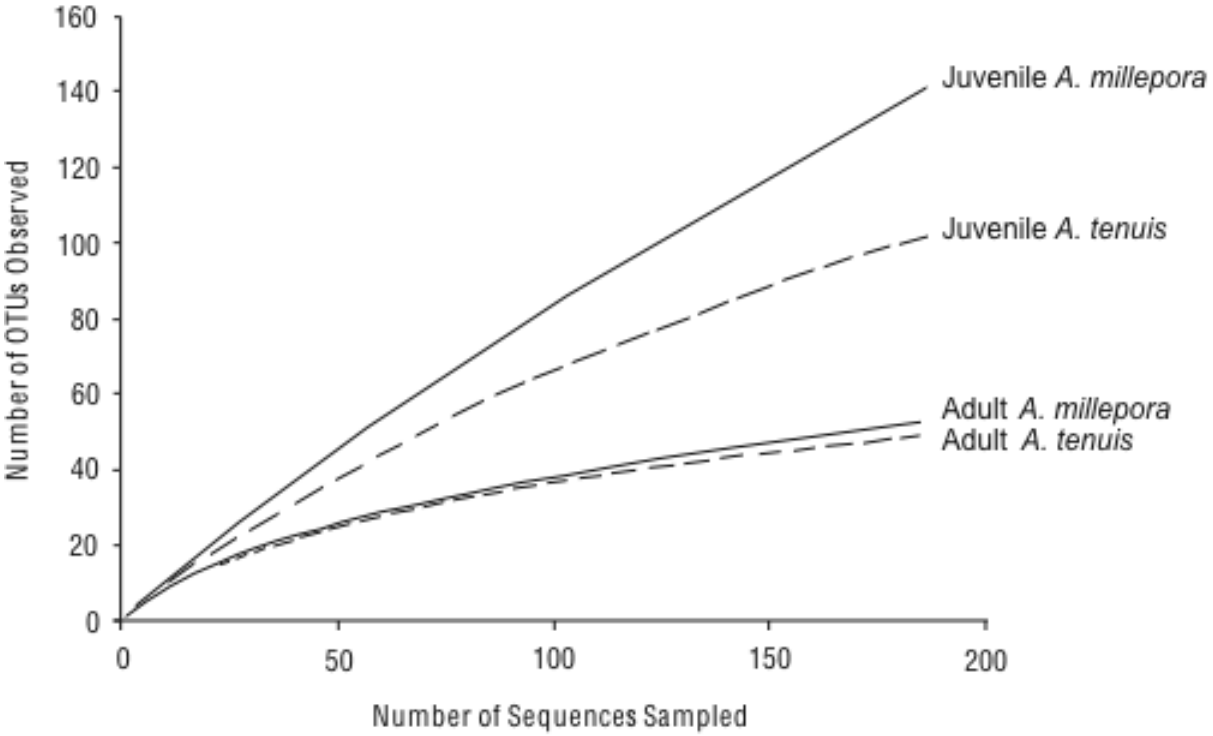

Figure 4.2 Rarefaction analysis of adult and 9-month juvenile *Acropora millepora* and *tenuis*. *A. millepora* corals compared harbor clade D while A. tenuis corals contain clade C1 *Symbiodinium*.

nMDS representation and direct comparisons of juvenile and adult coral bacterial DGGE profiles displayed tight grouping of bacterial fingerprints for adult corals of both *Acropora tenuis* (Figure 4.3a) and *A. millepora* (Figure 4.3b). Noticeably, the juvenile samples were widely spaced within the nMDS representation of DGGE fingerprints, indicating high variability in profiles in comparison to adult profiles. Similarly, nMDS plots of tRFLP profiles show much tighter grouping of adult coral samples relative to juvenile sample profiles for both species (Figure 4.3 c,d).

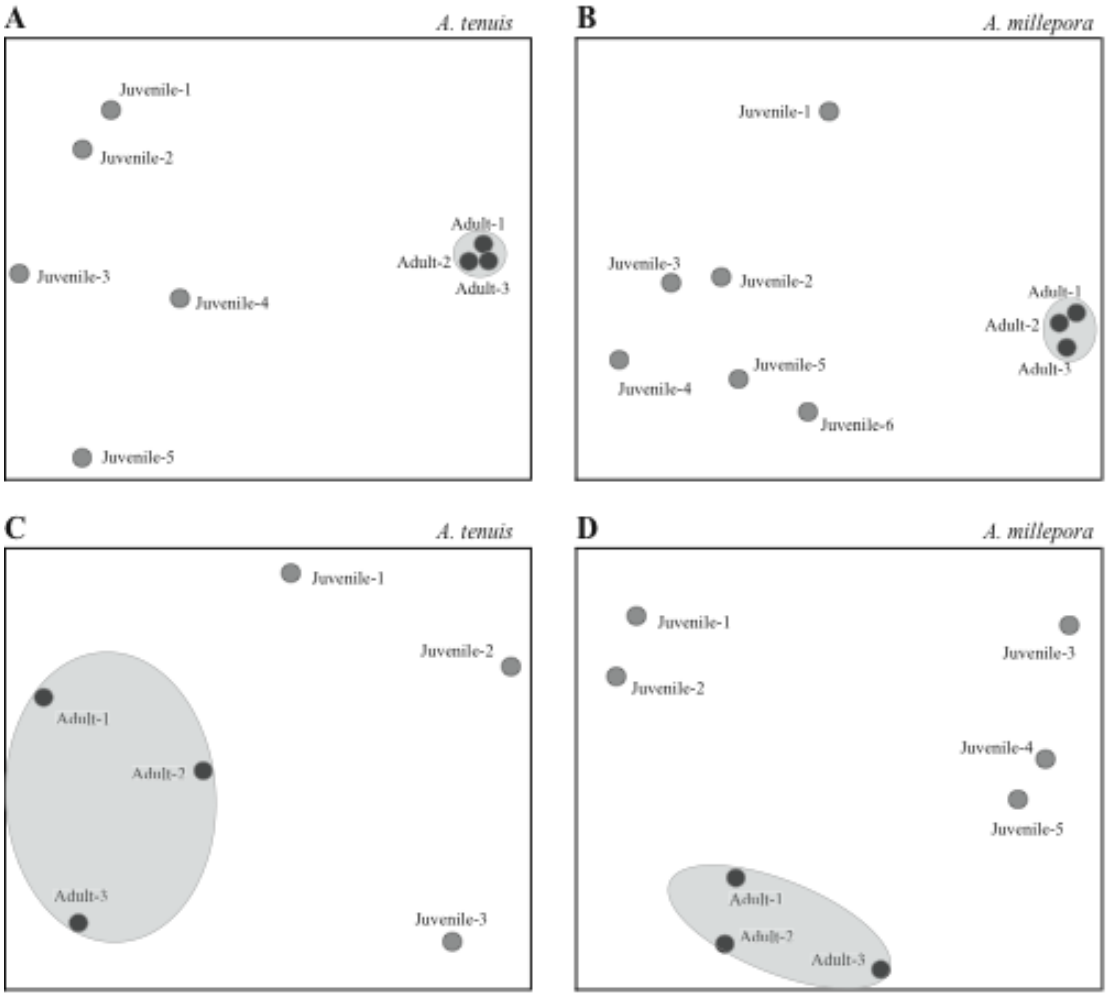

Figure 4.3 Comparison of 9-month juvenile and adult corals by nMDS of bacterial profiles. A.) DGGE profiles of *A. tenuis*. B.) DGGE profiles of *A. millepora*. C.) t-RFLP profiles of *A. tenuis*. D.) t-RFLP profiles of *A. millepora*.

Most sequences from adult *Acropora millepora* and *A. tenuis* clones libraries were closely related to sequences retrieved from juvenile libraries. For example phylogenetic analysis of dominant OTUs from juvenile libraries including OTUs 148 and 149 related to *Brevundimonas* sp., 313 related to *Spongiobacter* sp., 314 related to *Stenotrophomonas* sp.,168 related to *Roseobacter* sp. and 161 of the *Rhodobacter* group all correspond to dominant sequences retrieved from adult clone libraries (Figure 4.4). Therefore despite the high diversity and variability within juvenile libraries, many of the adult sequences are present in varying relative proportions,

although not consistently detected in all libraries (Table 4.2). Also, a few dominant sequences found on adult corals were not retrieved from juvenile samples, including the *δ-proteobacterium* (FJ489722), *Anaeromyxobacteria*-related sequence (FJFJ489711) and *Marinobacter*-related sequence (FJ489730).

4.3.3 Temporal changes in bacterial communities of A. tenuis

Survival of juvenile colonies of *Acropora tenuis* was reduced at 12 months (December 2007; Austral summer) when water temperatures reached 30^{o}C. At this sampling date, only 18 individuals (22%) from the *Symbiodinium* D treatment remained alive, compared to 81 colonies when sampled 3 months earlier. Colonies from the *Symbiodinium* D treatment exhibited little growth and average colony size remained at approximately 20 polyps (about 1 cm in diameter). In contrast, survival of *A. tenuis* from the *Symbiodinium* C1 treatment was four-fold greater (112 colonies or 89% remained alive) and colonies were noticeably larger in size (approximately 2-3cm) (Table 4.1).

Clone libraries of one-year old *A. tenuis* juveniles infected with different clades of *Symbiodinium* displayed similar proportions of bacterial classes, although a greater proportion of γ-*Proteobacteria* affiliated sequences were recovered from *A. tenuis* hosting clade D (33% and 34% of the sequences, respectively) in comparison to libraries of *A. tenuis* hosting clade C1 (11% and 17%). Bacterial profiles derived from clone libraries were analysed at the genus and family level using principal components analysis (PCA). The dominant ribotypes (grouped at 97% sequence identity) included in the PCA comprised >2% of any library compared and accounted for 88% of the variability (Figure 4.5). The PCA results for one-year old *A. tenuis* revealed one distinct difference between samples hosting clade D and clade C1 *Symbiodinium*. For

both *A. tenuis* libraries containing clade D, the sequences OTU-348 and 350 affiliating with *Vibrio* sp. were the dominant bacterial ribotype retrieved and displayed as a prominent vector on the PCA plot. However, less dominant

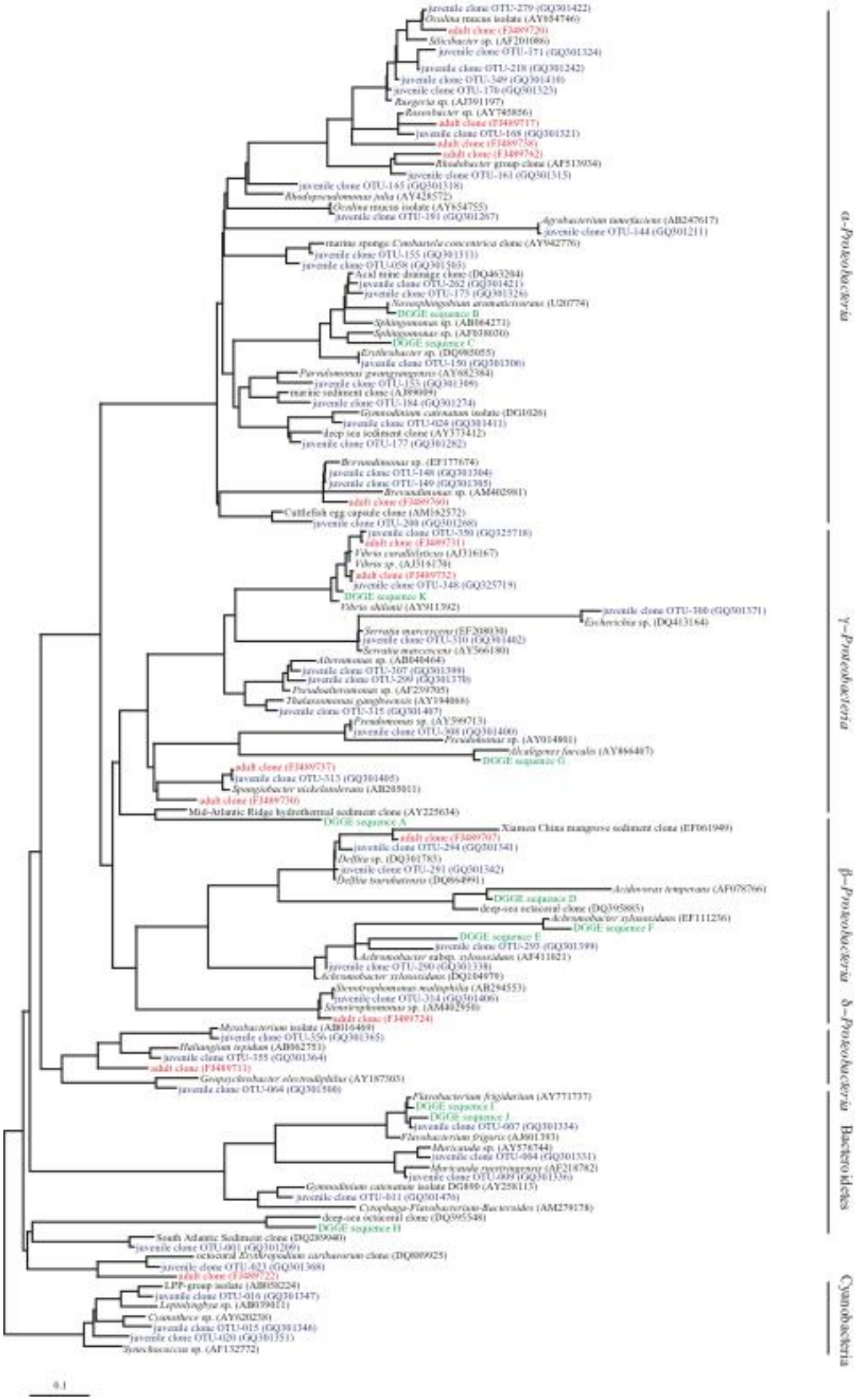

Figure 4.4 Identity of bacterial ribotypes that comprise >2% of juvenile 16S rRNA libraries or were retrieved from DGGE fingerprints. Tree topologies were evaluated by reconstructing phylogenies using evolutionary distance (Phylip Distance Method with

Jukes and Cantor model) analysis of aligned near full-length sequences (>1000 bp) (Ludwig et al. 1998).

sequences within the libraries separated the two *A. tenuis* clade D libraries on the PCA plot. The sequences retrieved from *A. tenuis* clade D library 1 affiliated with *Vibrio* sp. (13.9% of the library), *Pseudomonas* sp. (6.9% of the library), *Ruegeria* sp. (6.9% of the library) and *Roseobacter* sp. (4.2% of the library), while the dominant sequences retrieved from *A. tenuis* clade D library 2 affiliated with *Vibrio* sp. (18.8% of the library) and other *Bacteroidetes* (6.3% of the library) (Table 4.2). In contrast, no sequences affiliated with *Vibrio* sp. were retrieved from the *A. tenuis* libraries that contained clade C1 *Symbiodinium*. Variation within the dominant retrieved sequences of clade C1 corals also caused separation of libraries on the PCA plot. Dominant sequences within the *A. tenuis* clade C-1 library affiliated with *Brevundimonas* sp. (10.4% of the clone library), *Flavobacteria* sp. (8.3% of the library), *Shigella* sp. (6.3% of the library) and *Sphingomonas* sp. (8.3% of the library), whereas the sequences that were most prevalent in the *A. tenuis* clade C-2 library affiliated with *Flavobacteria* (5.3% of the library) and *Roseobacter* sp. (5.3% of the library) (Table 4.2). The *Vibrio* sp. affiliated sequences within the clade D *A. tenuis* libraries accounted for the 2 to 3-fold increase in the proportion of γ-*Proteobacteria* affiliated clones within these libraries compared to the clade D *A. tenuis* libraries. The *Vibrio*-like sequences OTU348 and 350 were closely related (99% identity) to the known coral pathogen *Vibrio coralliilyticus* (AJ316167) and *Vibrio* sp. (AJ316170) isolated from white syndrome (Figure 4.4).

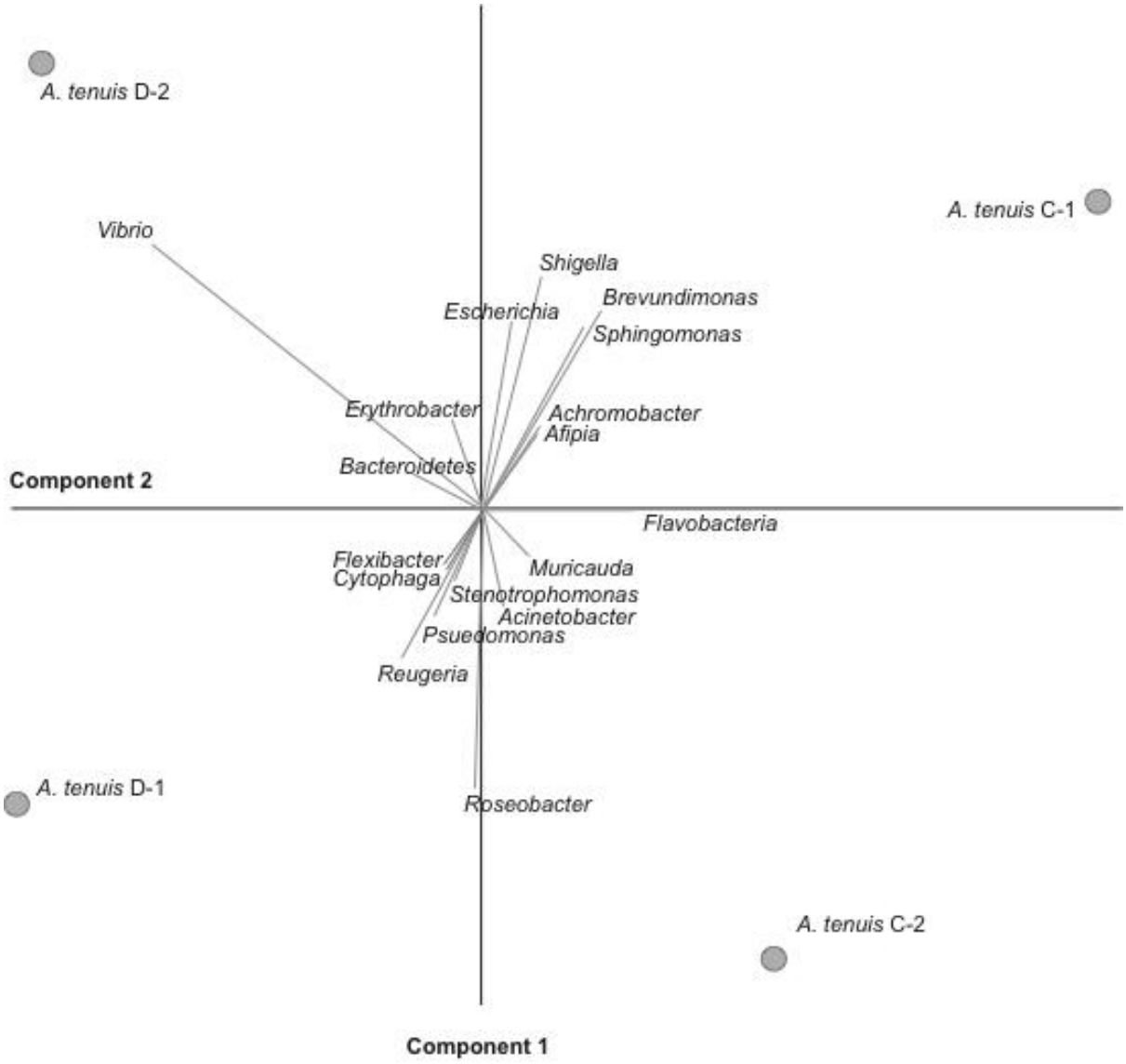

Figure 4.5 Principal components analysis of one-year juveniles harbouring clade C1 and D *Symbiodinium*.

Distinctly different DGGE bacterial banding patterns were observed for replicate 1-year samples of *A. tenuis* corals infected with D versus C1 *Symbiodinium* (Figure 4.6a). nMDS representation of these DGGE profiles demonstrated grouping of clade D corals (Figure 4.6b). The dominant band (Figure 4.6a; band K) within clade D profiles was related with *Vibrio* species including a *Vibrio* (98% sequence identity) isolated from white syndrome (EU372927) (Table 2, Figure 4.4), confirming observations that *Vibrio* sp. comprised a large component of the 1-year old juvenile clone libraries.

Interestingly the 1-year juveniles hosting clade C1 also appear to have profiles that are consistent between replicate samples with bands A-J (Table 4.2) present in all profiles (Figure 4.6a). This was in contrast to the profiles of 9-month juveniles, which lacked consistent dominant microbial associations.

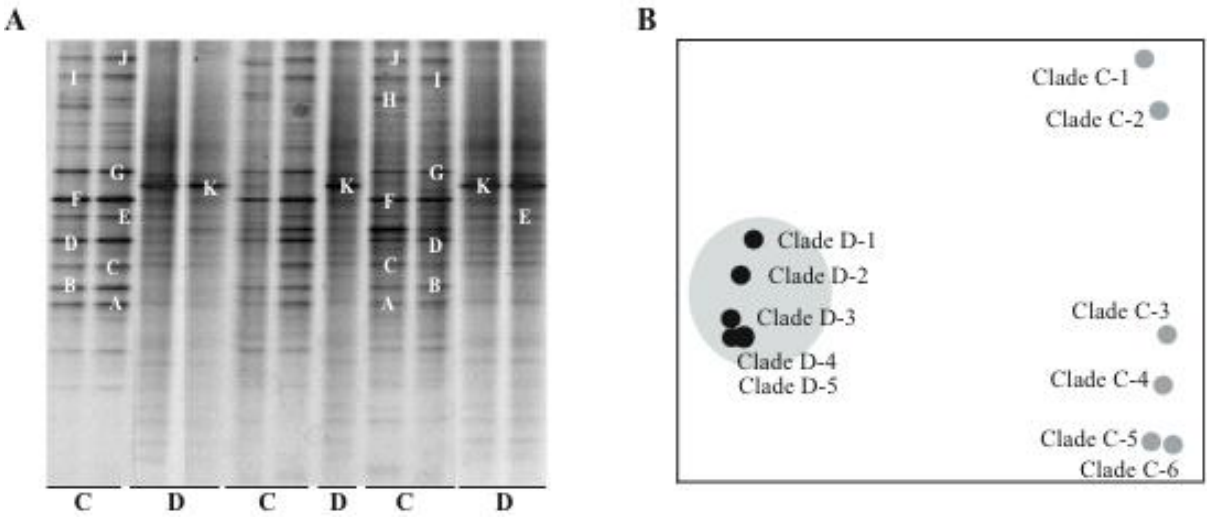

Figure 4.6 Denaturing gradient gel electrophoresis profiles for one-year *A. tenuis* harbouring clade C1 and D *Symbiodinium*. A.) DGGE gel of samples containing different clades. Bands labeled A-K. B.) nMDS plot of DGGE profiles.

Table 4.4 Affiliation of DGGE bacterial sequences retrieved from one-year juvenile coral colonies (n = 11)

Band	Closest relative and database accession number	Alignment (bp)	Similarity[c] (%)	Taxonomic description
A	Uncultured bacterium (DQ684483)	241/304	79	*γ –Proteobacteria**
B	Uncultured bacterium (EU808101)	292/294	99	*α-Proteobacteria**
C	*Sphingobium* sp. (EU679660)	270/295	91	*α-Proteobacteria*
D	Uncultured bacterium (EU172379)	300/303	99	*β-Proteobacteria**
E	Uncultured bacterium (EU535510)	270/309	87	*β-Proteobacteria**
F	*Achromobacter xylosoxidans* (EU877076)	274/285	96	*β-Proteobacteria*
G	*Alcaligenes* sp. (EU304282)	300/303	99	*β-Proteobacteria*
H	Uncultured bacterium (EU010191)	287/315	91	
I	*Flavobacterium frigidarium* (AY771738)	304/309	98	*Bacteroidetes*
J	*Flavobacterium frigidarium* (AY771738)	298/308	96	*Bacteroidetes*
K	*Vibrio* sp. (EU372935)	307/311	98	*γ -Proteobacteria*

[c] Sequences were aligned to the closest relative using BLAST (Altschul, 1997). The similarity was calculated with gaps not taken into account.

*Taxonomic description determined by phylogenetic analysis (Figure 4.4).

4.4 Discussion

4.4.1 Juvenile corals lack conserved microbial associates

Coral microbial investigations over the last decade have highlighted the important role that the host's microbial partners, including photosynthetic *Symbiodinium* (zooxanthellae) and associated bacterial, fungal and archaeal microbiota, play in coral health (Knowlton & Rohwer 2003). These studies have suggested that some adult corals conserve their bacterial partners, based on evidence that these corals harbor species-specific bacterial communities (Rohwer 2001; 2002). More recently it has been shown that, in some coral species, bacterial associations vary between reef locations (Littman et al. 2009) and the current study demonstrates that juvenile hosts do not establish the conserved microbial patterns characteristic of adult

corals at early development stages. Support for this latter conclusion was based on the absence of conserved patterns in bacterial communities using bacterial 16S rRNA gene profiles from 9-month old colonies of *A. tenuis* and *A. millepora* and three independent community profiling methods (clone libraries, DGGE and tRFLP analyses). Comparisons with a previous study of adults of the same coral species associated with the same *Symbiodinium* clades (Littman et al. 2009) highlight differences between juvenile and adult profiles, notably the highly conserved microbial profiles observed in adult corals using all three 16S rRNA gene profiling methods. Clone libraries of adults displayed lower relative diversity compared to juveniles, as determined by both diversity indices and rarefaction analysis, while nMDS interpretations of DGGE and t-RFLP profiles displayed tight grouping of adult coral profiles in contrast to no apparent relationship among juvenile coral profiles.

Results from this study also indicate that juvenile corals at 9-months of age do not show any discernable relationship between *Symbiodinium* clade present in the coral host and bacterial community structure. Previous research has demonstrated that genetically different *Symbiodinium* clades differentially affect host physiology, resulting in, for example, different heat tolerances or growth rates (Fitt 1985; Baker 2004; Little et al. 2004; Berkelmans & van Oppen 2006; Abrego et al. 2008). Since *Symbiodinium* partners exude substantial amounts of assimilated carbon into the mucus layer (Ikeda & Miyachi 1995), variation in *Symbiodinium* clades may affect the composition of coral mucus and subsequently select variant coral-associated microbiota, partly explaining species-specific associations in previous studies (Littman et al. 2009). However clone libraries, DGGE and tRFLP analyses all consistently showed no conserved diversity or specific associations for clade C1 versus D infected *A. tenuis* and *A. millepora* colonies at 9 months. We therefore conclude that the coral's

Symbiodinium clade is not a principal factor driving differences in microbial partners in early developmental stages of these two coral species and that at this early developmental stage, the high bacterial diversity found compared to adults indicates that no selection of bacterial partners is taking place. A lack of specificity for microbial associations in early ontogeny has also been observed in the initial uptake of different *Symbiodinium* types. For instance, the apparent specificity for strain C1 observed in adult populations of *Acropora tenuis* is not present in the early stages of infection (Little et al. 2004). New recruits take up both C1 and D strains and become dominated by *Symbiodinium* clade D after ~4 months, although type C1 eventually dominates, either through competition between different symbiont types or host mediated up-regulation (Little et al. 2004). It is noteworthy that the establishment of *Symbiodinium* partners in early ontogeny is a dynamic process and it can take up to three years to establish adult patterns of *Symbiodinium*-coral symbioses (Abrego et al. 2009).

It has been proposed that a lack of specificity for symbionts may serve as an adaptive mechanism for establishing associations with multiple symbionts that have different physiological characteristics (Little et al. 2004; Abrego et al. 2009). Similarly, multiple bacterial types may settle on new corals until coral-bacterial interactions lead to an established community best suited for particular environmental conditions. This may explain why certain ribotypes appear with different levels of dominance within the juvenile profiles, such as *Stenotrophomonas* and *Brevundimonas* related sequences, observed frequently in DGGE profiles and in higher proportions in some clone libraries. Furthermore, phylogenetic analysis of dominant clone sequences from both adult and juveniles showed some similarities. Many sequences retrieved from adult corals were also found in the 9-month juvenile libraries (Appendix 2), suggesting that the juveniles may be in the process of establishing their adult

associations. However, may sequences retrieved from adult corals were not detected in juvenile clone libraries, possibly suggesting a successional process whereby the diverse bacterial communities are gradually replaced by the adult associates, Such a winnowing process (Nyholm & McFall-Ngai 2004) is similar to patterns emerging for the establishment of *Symbiodinium* endosymbiosis (Rodriguez-Lanetty et al. 2006) and may reduce the comparatively high bacterial diversity of juveniles and result in the establishment of conserved adult bacterial communities. Apprill et al. 2009 similarly found distinct communities of bacteria associated with oocyte bundles, spawned eggs and planulae larvae of *Pocillopora meandrina*, supporting the observation that bacterial associates change throughout early developmental stages. However, planulae larvae only internalized one *Roseobacter* clade after 79 hours indicating that coral can have specificity for associating with certain bacteria early in development. Future studies are required to examine the dynamics of bacterial associations throughout the full ontogeny of corals from larval stage until adulthood to monitor the progression and establishment of a stable species-specific bacterial community.

4.4.2 Temperature stress on coral juvenile bacterial associations

The greater mortality rates of *A. tenuis* juveniles hosting clade D *Symbiodinium* (78%) in comparison to C1-juveniles (22% mortality), plus their three-fold smaller sizes (1 cm diameter for D-juveniles versus 3cm diameter for C1-juveniles) (Table 4.1) indicate that *A. tenuis* juveniles harbouring clade D were less fit and potentially received fewer nutrients for growth. Growth rates observed in this study are supported by those of Little et al. (2004) that showed a 2-3 fold higher growth rate in *A. tenuis* and *A. millepora* harbouring clade C1 compared to D. Clone libraries derived from *A. tenuis* juveniles harbouring clade D possessed, in contrast to clade C1 juveniles, a higher proportion of clones aligned with *γ-Proteobacteria*, with sequences related to

Vibrio species constituting the majority of these clones. The DGGE bacterial profiles similarly demonstrated the prominence of *Vibrio* sp. related sequences in *A. tenuis* samples hosting clade D juveniles that were not apparent in clade C1 juveniles. The appearance of *Vibrio*-like sequences as dominant members of the microbial communities in the seemingly unhealthy clade D samples is noteworthy given that these organisms are often regarded as opportunistic pathogens in marine systems (Saeed 1995; Li et al. 1999; Kraxberger-Beatty et al. 2006), with some *Vibrio* species implicated specifically as coral pathogens (Kushmaro et al. 1997; Ben-Haim & Rosenberg 2002; Ben-Haim et al. 2003; Sussman et al. 2008). Moreover, *Vibrio*-like sequences retrieved from 1-year clade D samples closely aligned with *Vibrio coralliilyticus* (Supplementary Figure 4.1), which has been demonstrated as a temperature-dependent pathogen, exhibiting tissue lysis of *Pocillopora damicornis* (BenHaim and Rosenberg 2002; Ben-Haim et al 2003). Seawater temperatures were between 29-30°C at the time of sampling, indicating that the corals may have been experiencing thermal stress, increasing their susceptibility to pathogenic infection. For example, it has been demonstrated that *Vibrio shiloi* can switch on temperature regulated virulence factors at 28°C enabling bacterial infection and coral bleaching (Kushmaro et al. 1997; Ben-Haim et al. 1999; Banin et al. 2001). One study examined bacterial communities associated with corals through a bleaching event and demonstrated a relative increase in *Vibrio* related sequences prior to and during the peak stress of the bleaching period (Bourne et al., 2008). However, *Vibrio* sp. have also been shown to be a normal constituent of the bacterial community associated with healthy coral (Littman et al. 2009) and the nitrogen-fixing capabilities of coral-associated *Vibrio* may be important for the nitrogen cycling within the holobiont (Olson et al. 2009). Therefore, it is likely that *Vibrio* sp. normally reside on the surface

of the coral with environmental stressors such as higher seawater temperatures changing the homeostasis of coral microbial associates, permitting proliferation of these organisms. Nevertheless, the deterioration of coral juvenile health and correlation with *Vibrio* dominance in clone libraries as well as frequency in DGGE bacterial profiles implies that these organisms potentially play an important role in coral health. Further controlled temperature stress experiments utilizing corals harboring different *Symbiodinium* clades are required to establish whether the entire holobiont is more stable with certain types of *Symbiodinium* and how *Vibrio* proliferation affects overall coral health.

The decreased fitness of corals hosting clade D observed in this study has greater implications for coral resilience to climate change. Previous studies have suggested a higher tolerance to heat for corals associated with type D (Glynn et al. 2001; Toller et al. 2001; Baker et al . 2004; Fabricius et al . 2004; Rowan 2004; Berkelmans & van Oppen 2006). For example Berkelmans and van Oppen (2006) demonstrated that *Acropora millepora* shifted the dominant symbiont from clade C2 to D with subsequent experiments showing that corals were more thermally tolerant when hosting clade D. However, these studies involved species in which type D is homologous. Abrego et al. (2009) showed that *A. tenuis* in which type C1 is homologous, were more thermally tolerant with clade C1 than with clade D suggesting that host factors are also involved in determining heat tolerance. Our results are consistent with this conclusion, indicating clade D *A. tenuis* may be more susceptible to bacterial proliferation under heat stress. Two studies have speculated that *Symbiodinium* type may contribute to coral resistance to disease, providing possible explanations for differences in coral communities hosting different clades. Sussman et al. 2009 showed differential susceptibility of *Symbiodinium* clades to a

metalloprotease, suggesting that coral (specifically *A. millepora*) may be more susceptible to PSII inactivation when hosting clade A. However, corals harbouring clade C1 and D did not show differences in this regard. Stat et al. 2008 found sub-optimal health states of *A. cytherea* harboring clade A as well as increased incidence of disease compared with coral harbouring clade C. In addition, they demonstrated significantly higher amounts of carbon released by clade C than clade A, providing evidence for less nutrients passed on to the host and therefore decreased fitness of the host as a mechanism for disease susceptibility. The smaller sizes of juveniles hosting clade D indicate less nutrient acquisition than those hosting clade C1, suggesting a trade-off for corals when hosting clade D versus C1. Further investigations are required to find possible consequences of corals shifting *Symbiodinium* type.

4.4.3 Conclusions

We conclude that colony age, heat stress and potentially *Symbiodinium* type may contribute to the establishment and dynamics of bacterial communities associated with the coral holobiont. High and variable bacterial diversity on juvenile corals, as indicated by clone libraries, DGGE and tRFLP analysis, suggest that a winnowing process takes place throughout early developmental stages of juvenile coral growth to establish adult patterns of species-specific bacterial associations. Lower growth rates and higher mortality rates of D-juveniles of *A. tenuis* indicate that they are less fit than C1-juveniles. Concomitant proliferation of *Vibrio* sp. on corals harbouring clade D suggests that coral associated with *Symbiodinium* type C1 may be less susceptible to opportunistic pathogens during times of environmental stress. Therefore, it is possible that the *Symbiodinium* clade may be important for supplying corals with enough energy to ensure a stable microbial community when subjected to elevated temperatures.

Chapter 5.0 Responses of coral-associated bacterial communities to heat stress differ with Symbiodinium type on the same coral host

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All the data was collected and analyzed by R. Littman, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.

5.1 Introduction

There has been steady growth in knowledge of the dynamics of coral-algal endosymbioses in the past decade (e.g. Brown 1997, Jones et al. 1998, Lesser 2006, Weis 2008, van Oppen et al. 2009), but less is known about the dynamics of other microbial organisms involved in coral symbioses. It is well established that the dinoflagellate partnership is integral to the health of the coral holobiont and loss of *Symbiodinium* (also known as zooxanthellae) and the photosynthates that they translocate typically constitutes a nutritional crisis for the coral host (Brown 1997, Grotolli et al. 2006). However, reef-building corals are holobionts that encompass not only the coral animal and its algal endosymbiont, *Symbiodinium,* but also various microbes, including, bacteria, fungi, *Archaea* and viruses (Knowlton and Rohwer 2003). To develop a holistic understanding of coral health, greater understanding of the consequences of changes in the composition of bacterial communities to the coral holobiont is needed.

Elevated seawater temperature is the prime factor responsible for geographically widespread disruptions to *Symbiodinium*-coral symbioses, which can manifest as mass coral bleaching events, generally as a consequence of reactive oxygen species damaging photosynthetic and mitochondrial membranes (Weis 2008). The adaptive bleaching hypothesis posits that bleaching may be a mechanism to enhance the fitness of reef-building corals, by either switching or shuffling less heat tolerant for more heat tolerant *Symbiodinium* types, thereby enabling adaption to increasing seawater temperatures (Buddemeier and Fautin 1993, Baker 2003). Studies demonstrating shifts in *Symbiodinium* types associated with corals that have recovered from bleaching events provide support for this hypothesis (Berkelmans and van Oppen 2006, Jones et al. 2008).

Just as *Symbiodinium*-coral symbioses have been shown to be flexible, coral-associated bacterial communities may also respond to environmental conditions. The coral probiotic hypothesis suggests that selection for microbial symbiotic partners could enhance the fitness of the coral holobiont and that microbial associations are likely to represent dynamic interactions with ambient environmental conditions (Reshef et al. 2006). However, the effect of elevated temperature on bacterial communities associated with corals has not been fully explored. A field-based study found a correlation between a coral bleaching event and the appearance of *Vibrio* affiliated sequences, suggesting that heat stress can result in shifts away from conserved healthy bacterial communities and potential proliferation of opportunistic pathogens (Bourne et al. 2008). Ritchie (2006) found that coral mucus samples collected during a bleaching event lacked antibiotic activity characteristic of mucus collected from healthy corals, suggesting a shift away from beneficial bacterial assemblages under environmental stress. Similarly, it has been found that proliferation of *Vibrio* species coincides with heat stress in controlled temperature experiments (Ainsworth and Hoegh-Guldberg 2009) and moreover, subtle metabolic changes in *Vibrio* species under heat stress, even when in low abundance, can cause major shifts in the metagenomes of coral associates (Vega-Thurber et al. 2009). Given the major impact that elevated temperatures have on the *Symbiodinium*-coral symbiosis, both in terms of partial and whole colony mortality of the coral holobiont, and in light of predictions for increased frequency and intensity of coral bleaching events (Brown 1997, Hoegh-Guldberg 1999, Hoegh-Guldberg 2004), it is timely to consider how coral-associated bacterial communities may change with heat stress in combination with interactions between *Symbiodinium* type and the coral host.

Symbiodinium types can interact differentially with coral hosts to yield differences in holobiont physiology that may have indirect effects on microbial diversity. For example, some corals are more tolerant to heat stress when hosting *Symbiodinium* ITS 1 type D (Baker 2004, Berkelmans and van Oppen 2006), whereas Abrego et al. (2008) demonstrated that *Acropora tenuis* is more heat tolerant in association with ITS 1 type C1, indicating that host factors also interact with *Symbiodinium* type to determine holobiont physiology. Furthermore, both *A. tenuis* and *A. millepora* juveniles grow 2-3 times faster when associating with *Symbiodinium* ITS 1 type C1 than with *Symbiodinium* D (Little et al. 2004). Differential growth of the coral holobiont may result from a greater contribution of C1 *Symbiodinium* to host nutrition, both through faster rates of population growth inside the host (Fitt 1985) and greater translocation of photosynthates to coral tissues (Cantin et al. 2009). Such differences in the nutritional economy of the coral host are likely to have major effects for coral mucus production and hence the microbial communities that live in the surface mucus layer (SML). For instance, most (as high as 98%) of the net carbon assimilated by *Symbiodinium* is released as exudates into the mucus (Ikeda and Miyachi 1995), affecting its biochemical composition, with potentially important implications for structuring microbial communities. Ritchie and Smith (1997) demonstrated that selective carbon source utilization by cultured bacteria differs among 11 coral species. Variation in photosynthetic contributions by different *Symbiodinium* types could therefore affect the composition of coral mucus, indirectly impacting the coral microbiota. Initial comparisons of *A. tenuis* juveniles hosting ITS 1 types C1 and D did not reveal any discernable differences in bacterial associates at 9 months (Littman et al 2009b). Given the greater bacterial diversity associated with juveniles in comparison to adults in this study, these patterns are consistent with non-

specific bacterial colonization of the SML early in ontogeny, as has been found for *Symbiodinium* uptake in this coral species (Abrego et al. 2009). Nevertheless, examining corals hosting different symbiont types in times of environmental stress may reveal differences in vulnerabilities in bacterial communities. For instance, a recent study found differential susceptibility of *Symbiodinium* ITS 1 types to zinc-metalloprotease activity of the bacterial pathogen, *Vibrio coralliilyticus* (Sussman et al. 2009) indicating that holobionts associating with different *Symbiodinium* types may differ in their interactions with some bacterial species.

This study aimed to 1) characterize bacterial communities associated with juveniles of the coral, *Acropora tenuis*, hosting different dominant algal symbionts (ITS 1 types C1 and D), and 2) determine whether bacterial communities associated with juvenile corals dominated by different algal endosymbionts are differentially susceptible to thermal stress. Juvenile corals were raised from larvae to enable manipulation of the *Symbiodinium* type harboured upon initiation of symbiosis, and were experimentally infected with either *Symbiodinium* ITS 1 type C1 or D.

5.2 Materials and methods

5.2.1 Gamete collection, larval settlement and Symbiodinium infection

Juveniles of the coral, *Acropora tenuis*, were raised from larvae and experimentally infected with *Symbiodinium* ITS 1 type C1 or D as described in Abrego et al. (2008). Parental colonies were collected from the upper reef slope in Nelly Bay, Magnetic Island (19º10's 146º50'E) just prior to spawning in November 2006 and gametes were collected and combined from four colonies to produce larvae. Following settlement of larvae and infection with the appropriate algal symbiont, juvenile corals were returned to the parental habitat, where they remained until collection. Tiles with

attached juvenile corals were placed on steel rods and suspended vertically between pairs of metal star-pickets on the reef flat in Nelly Bay, Magnetic Island, where they were allowed to grow for twelve months. Hereafter, coral juveniles infected with ITS 1 C1 or D *Symbiodinium* types will be referred to as C1- or D-juveniles, respectively.

5.2.2 Temperature stress experiment

Tiles were collected from the reef flat in December 2007, when seawater temperatures were ~30°C ±0.5°C (based on AIMS temperature logger records of average ambient seawater temperatures at the site), and placed in bins for transport to controlled aquaria facilities at Orpheus Island Research Station. Juvenile colonies were sampled prior to removal from the reef to provide a baseline for coral-associated bacterial diversity and to determine if bacterial communities shifted after placement in aquaria held at typical summer temperatures (28°C; designated as the control treatment). Excess algae were cleaned from tiles, which were then placed in flow-through aquaria with one micron filtered and UV treated seawater. Water was fed from larger temperature-controlled sumps into smaller replicate aquaria to ensure all juveniles experienced the same seawater conditions. Tiles containing C1- and D-juveniles were placed randomly into 6 aquaria (3 control aquaria and 3 experimental aquaria). All corals were acclimated at 28°C for three days prior to the experiment. One day before the start of the experiment, the temperature within the experimental aquaria was ramped up 0.5°C every six hours until the water temperature reached 32°C and then maintained at this target temperature. Seawater temperatures experienced by local reef flat corals commonly change at rates of more than 1°C per hour during spring tides (Berkelmans & Willis 1999), much greater than the increment used in this study. Control aquaria were held at 28°C throughout the experiment. Irradiance levels were set at 360µmol photon m^{-2} s^{-1}, as described in Abrego et al (2008). Temperatures

of each tank were monitored using a digi-therm thermometer accurate to 0.2°C and the maximum deviation from target temperatures was found to be between 0.2 °C and 0.5°C.

The experiment was carried out for 14 days and the elevated temperature treatment was set at 32°C. *Acropora* species generally bleach when seawater temperatures equal or exceed 32°C, which is typically only 2-3°C higher than average seawater temperatures during summer at Orpheus Island (Berkelmans and Willis 1999). High mortality rates of type D juveniles in the field resulted in a smaller avaliable experimental sample size (n=20) relative to C-juveniles and therefore sampling of D-juveniles could only be carried out until day 12 before all samples were exhausted. At designated sampling times, the health status of each juvenile in the control and elevated temperature treatments was assessed macroscopically Then two replicate juvenile colonies were collected for each sample type (*A. tenuis* infected with either ITS 1 C1 or D *Symbiodinium*) from within each treatment, i.e. control (28°C) and elevated temperature treatments (32°C). Two juvenile colonies were also sampled for each ITS 1 type in the field prior to experimentation, which were named "*in situ"* samples. Whole juveniles colonies were sampled from the settlement tiles by detaching colonies with a sterile scalpel, rinsed with artificial seawater and placed in cryovials and stored at -80ºC.

5.2.3 Photochemistry

The efficiency of photochemical reactions associated with photosynthesis of the algal endosymbiont was measured with a pulse amplitude modulated fluorometer (PAM) and maximum quantum yield of PSII (Fv/Fm) used as an indicator of heat stress. A diving-PAM (diving-PAM, Walz) was fitted with a 2 mm diameter fibre

optic probe and measurements were taken for 8 replicate C1-juveniles for each treatment. To maintain consistent replication of measurements throughout the experiment, only 2 replicate D-juveniles were measured for each treatment at each sampling time because of the limited number of surviving individuals (exhausted by day 12). Fluorescence measurements were taken every second day and measurements of dark-adapted colonies were recorded during the morning before lights were turned on.

5.2.4 DNA extraction and purification

DNA was extracted by suspending an entire juvenile colony in 0.5 ml of buffer (0.75M Sucrose, 40mM EDTA, 50mM Tris, pH 8.3) and following the extraction protocol outlined in Bourne et al. (2008). The DNA pellet was suspended in 30 µl of sterile milli-Q water and the total volume was loaded onto a 1.2% low-melting agarose gel. Gel separation has been found to improve subsequent PCR amplification; therefore DNA was purified by using electrophoresis and cutting high quality DNA (>2kb) from the gel. The agarose was then removed from the sample by using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA was recovered from the Qiagen column with two 30µl washes of sterile milli-Q water.

5.2.5 PCR amplification of 16S ribosomal RNA gene

Universal primers 63f and 1387r (Marchesi et al. 1998) were used to amplify the 16S rRNA genes from extracted DNA for bacterial clone library construction. PCR was performed with an Applied Biosystems 2720 thermocycler and programmed with an initial 3-min step at 94°C and 35 cycles consisting of 94°C for 1 min, 55°C for 1 min and 72°C for 1.5 min, and a final extension for 10 min at 72°C as described in

Chapter 2.2.3. For denaturing gel electrophoresis, the bacterial 16S rRNA gene was amplified using primers 1055f and 1392R-GC (Ferris et al. 1996). Temperature cycling was performed using a touchdown protocol (Ferris et al., 1996) with one cycle of 95°C for 15 min, 10 cycles of 94°C for 1 min, 53°C (each cycle decreasing by 1°C) for 1 min and 72°C for 1 min, followed by 20 cycles of 94°C for 1 min, 43°C for 1 min and 72°C for 1 min.

5.2.6 Single stranded conformation polymorphism (SSCP)

SSCP was used to identify the *Symbiodinium* type present in each juvenile sampled at each sampling time (n=18 D-juveniles and 22 C1-juveniles sampled in total) to ensure that corals still harboured the same C1 or D *Symbiodinium* type with which they were initially infected. The 18S Internal Transcribed Spacer 1 (ITS1) region of *Symbiodinium* was amplified using ITSF and ITSR primers (van Oppen et al. 2001). PCR products were prepared and run on acrylamide gels as described in section 4.2.5. Gels were imaged using Gel-Scan 3000 software (Corbett Robotics, Sydney, NSW, Australia).

5.2.7 Clone library construction

Two replicates of each C1- and D-juvenile type were collected at the following designated time points: immediately prior to removal of tiles from the field ("*in situ*"); 1 day after target temperatures were reached (T=1); and at the end of the experiment, i.e. either following 12 days in temperature treatments (T=12) for D-juveniles or following 14 days of treatment (T=14) for C1-juveniles. Juveniles collected on days 1, 12 and 14 of the experiment were sampled from each of the 28°C and 32°C treatments. Bacterial 16S rRNA genes amplified from DNA extracted from each individual juvenile were ligated into the TOPO®-cloning vector following the manufacturer's

instructions (Invitrogen, Carlsbad, CA, USA). Ligations were submitted to the Australian Genome Research Facility for transformation, cloning (96 clones per library) and subsequent sequencing. Replicate libraries for each sample type and time points were pooled to simplify statistical analysis. Dominant bacterial 16S rRNA gene sequences retrieved from each clone library were analysed using principal components analysis (PCA) to determine which sequences (affiliated at the genera level) were important in driving differences between the libraries. PCA analysis was carried out using PAST software program (Hammer et al. 2001). The variance-covariance option was chosen because all data were measured as the same unit (proportion of clone library). The 'View scatter' option was chosen to visualise data points plotted in the coordinate system given by the two most important components and the plot was based on an Euclidean distance measure of the original data points. Because of the high diversity of clone sequences retrieved, only sequences comprising 3% or greater (up to 40%) of each library were included in the PCA analysis in order to facilitate detection of the main trends while maximizing the number of clone sequences used and accounting for as much of the variance in the multidimensional data as possible.

5.2.8 Denaturing gradient gel electrophoresis analysis (DGGE)

Two replicate coral juveniles were collected on the following days, which were prior to or after target temperatures had been achieved: "*in situ*" (defined above), T=1, T=5, T=9 and T=12 for both D and C1 juveniles, along with additional C1 juveniles at T=14. Juveniles collected on days 1, 5, 9, 12 and 14 of the experiment were also sampled from each of the 28°C and 32°C treatments, which constituted a total of n=16 individuals for D-juveniles and n=20 individuals for C1-juveniles, in addition to two samples of juveniles associating with each *Symbiodinium* type from *in situ* field

samples (30°C). Bacterial profiling was carried out using an INGENY phorU-2 (Ingeny International BV, Netherlands) DGGE system. PCR products were separated on gels as described in section 2.2.5. Clear bands were excised from the gel and placed in 100 µl of sterile milli-Q water to elute DNA from the acrylamide gel. The DNA bands were re-amplified and run on the DGGE gel to ensure correct migration and purity of the product. Products that showed one distinct band with the correct mobility on the DGGE were directly sequenced. Using the sequences recovered from cut bands, a presence/absence matrix was constructed for the DGGE fingerprints and analyzed by non-metric multi-dimensional scaling (nMDS) using Euclidian distances. The nMDS two-dimensional plot was created using PAST software program (Hammer et al. 2001).

5.2.9 Sequence analysis

Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak et al. 1996). Sequence data were aligned to the closest relative using the BLAST database algorithm (Altschul et al. 1997). Sequence affiliations were determined by >97% identity to bacterial 16S rRNA gene sequences in the GenBank database under accession codes GU174642-GU174742.

5.3 Results

5.3.1 Aquarium experiments

A total of 112 C1-juveniles and 20 D-juveniles survived the twelve-month field grow-out period and were available for distribution amongst aquaria for experimental treatments. Fewer D-juveniles were available because of extensive mortality throughout the year, although the remaining live juveniles appeared visually healthy

and PAM measurements indicated that their photochemical efficiencies did not differ from those of C1-juveniles when held at 28°C. SSCP confirmed that all juveniles still harboured the ITS 1 type *Symbiodinium* with which they were originally infected. In the elevated temperature treatment, macrosopic signs of thermal stress were first noted on day 9 for D-juveniles, and all were white by day 12. In contrast, C1-juveniles first showed macroscopic signs of tissue paling in the elevated temperature treatment on day 11 and most remained pale (rather than white) until the experiment ended on day 14. PAM measurements also indicated that D-juveniles of *A. tenuis* were more thermally stressed than C1-juveniles at elevated temperatures. Because of the limited number of D-juveniles, photochemical efficiencies were only measured up to day 10 of the experiment, however these juveniles displayed a significant drop (44% decline) in maximum quantum yields when compared to controls on day 10 ($t = 13.44$, $p < 0.005$, t-test, Figure 5.1). In contrast, C1-juveniles displayed only a 10% decrease in F_v/F_m values by day 14, although this represented a significant reduction relative to C1-juveniles in the control treatment ($t = 1.09$, $p < 0.005$, t-test, Figure 5.1). No visual signs of tissue paling were observed for any of the C1- or D-juveniles in the control (28°C) treatment throughout the experiment. Furthermore, there were no significant differences in F_v/F_m for corals held at 28°C throughout the two-week duration of the study for either *Symbiodinium* type, indicating that the aquarium environment did not affect photochemical efficiency of the corals.

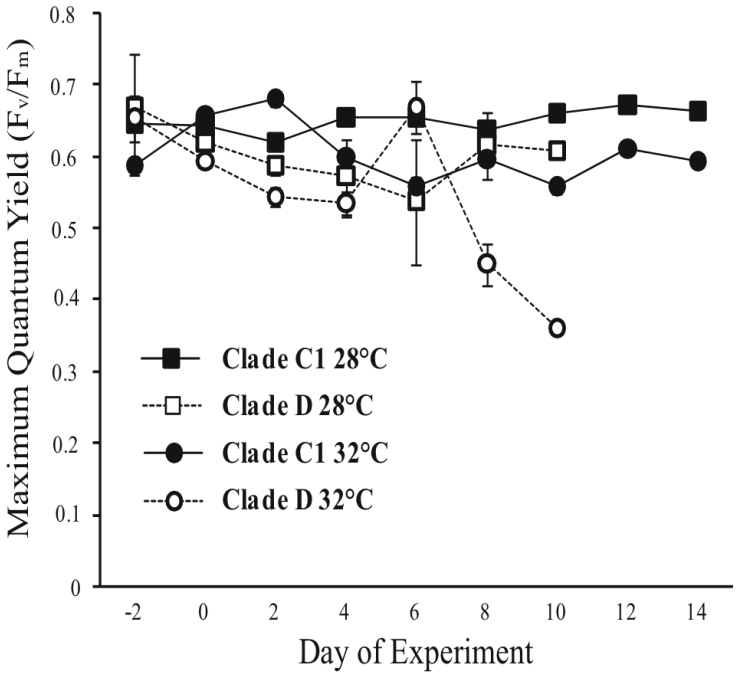

Figure 5.1 Maximum quantum yield (F_v/F_m) of corals hosting either *Symbiodinium* ITS1 type C1 or D at 28 and 32°C. Maximum quantum yield was used as a proxy for photochemical efficiency of photosystem II. Values are means ±s.e. for each *Symbiodinium* type.

5.3.2 Clone libraries

Bacterial 16S rRNA clone libraries derived from both C1- and D-juveniles were highly diverse, though dominated by *α*- and *γ-Proteobacteria* affiliated sequences. Dominant sequences within each library (those constituting >3% of each library) that had sequence identities of >97% were grouped into operational taxonomic units (OTUs) and classified to class and genera level based on BLAST identities. OTUs were represented in a principal component analysis (PCA), which explained 75% of the variation in the clone libraries (Figure 5.2). C1-juveniles sampled from the field (*in situ*) and after 14 days (T=14) at 28°C (control treatment) displayed similar

sequence profiles, indicating that placing the corals within aquaria did not affect associated bacterial communities. *In situ* sequences retrieved from the juvenile libraries were affiliated with a mixture of commonly associated coral microbiota, including *Roseobacter* and *Achromobacter,* as well as *Erythrobacter* and *Mesorhizobium* species. In the elevated temperature treatment, C1-juvenile libraries at day one (T=1) had similar bacterial associates but were dominated by *Brevundimonas, Sphingomonas, Shigella* and *Flavobacteria* affiliated sequences, causing a slight separation from *in situ* samples of C1-juveniles in the PCA plot. After 14 days at 32°C, the bacterial profiles of C1-juveniles remained similar to C1-juvenile samples in the control treatment, with only minor variation. The dominant bacterial associates included *Acinetobacter* sp., *Flavobacteria* sp., *Reugeria* sp., *Stenotrophomonas* sp., *Agrobacteria* sp., *Roseobacter* sp. and *Rhodobacteraceae* related sequences.

The clone library of D-juveniles sampled directly from the reef flat contained a bacterial community that differed from that of C1-juveniles sampled from the reef flat at the same time (Figure 5.2). Although many similar sequences were present in the two libraries (such as *Brevundimonas* sp., *Mesorhizobium* sp., *Roseobacter* sp. and *Stenotrophomonas* sp.), sequences affiliated with *Vibrio* sp. represented 13% of the *in situ* library for D-juveniles, but were absent from the *in situ* library of C1-juveniles (Figure 5.2). The presence of *Vibrio* sp. related sequences was a major factor driving separation of D- and C1-juveniles in the PCA plot (Figure 5.2). For D-juveniles maintained at 28°C for 12 days, the bacterial community shifted to become similar to communities associated with C1-juveniles sampled both from the field (i.e. *in situ*) and the two temperature treatments at T=14 (Figure 5.2). Interestingly, the relative proportion of *Vibrio* sp. sequences for the D-juvenile clone library in the control temperature treatment decreased to only 2% of retrieved clones at T=12 days (Figure

5.3). In contrast, libraries of D-juveniles in the elevated temperature treatment became dominated by *Vibrio* sp. affiliated clones, the percent of *Vibrio* sp. affiliated sequences increasing from 18% in the day 1 (T=1) library to 39% by day 12 (T=12) (Figure 5.3). The clear separation of D-juvenile samples along the long *Vibrio* sp. vector in the PCA plot highlights the comparative increase in the number of *Vibrio* sp. sequences in these samples by the end of the experiment (Figure 5.2). However, the D1-juvenile sample retrieved at day 1 (T=1) from 32°C was also strongly dominated by *Escherichia* sp. affiliated sequences, which comprised 28% of the clone library and caused a separation from the *in situ* and T=12 samples along the second axis of the PCA plot (Figure 5.2). Interestingly, the dominant sequence retrieved from clone libraries of D-juveniles in the elevated temperature treatment displayed highest sequence similarity to the *Vibrio coralliilyticus* 16S rRNA gene sequence, an organism previously implicated in coral bleaching and disease (Sussman et al. 2008).

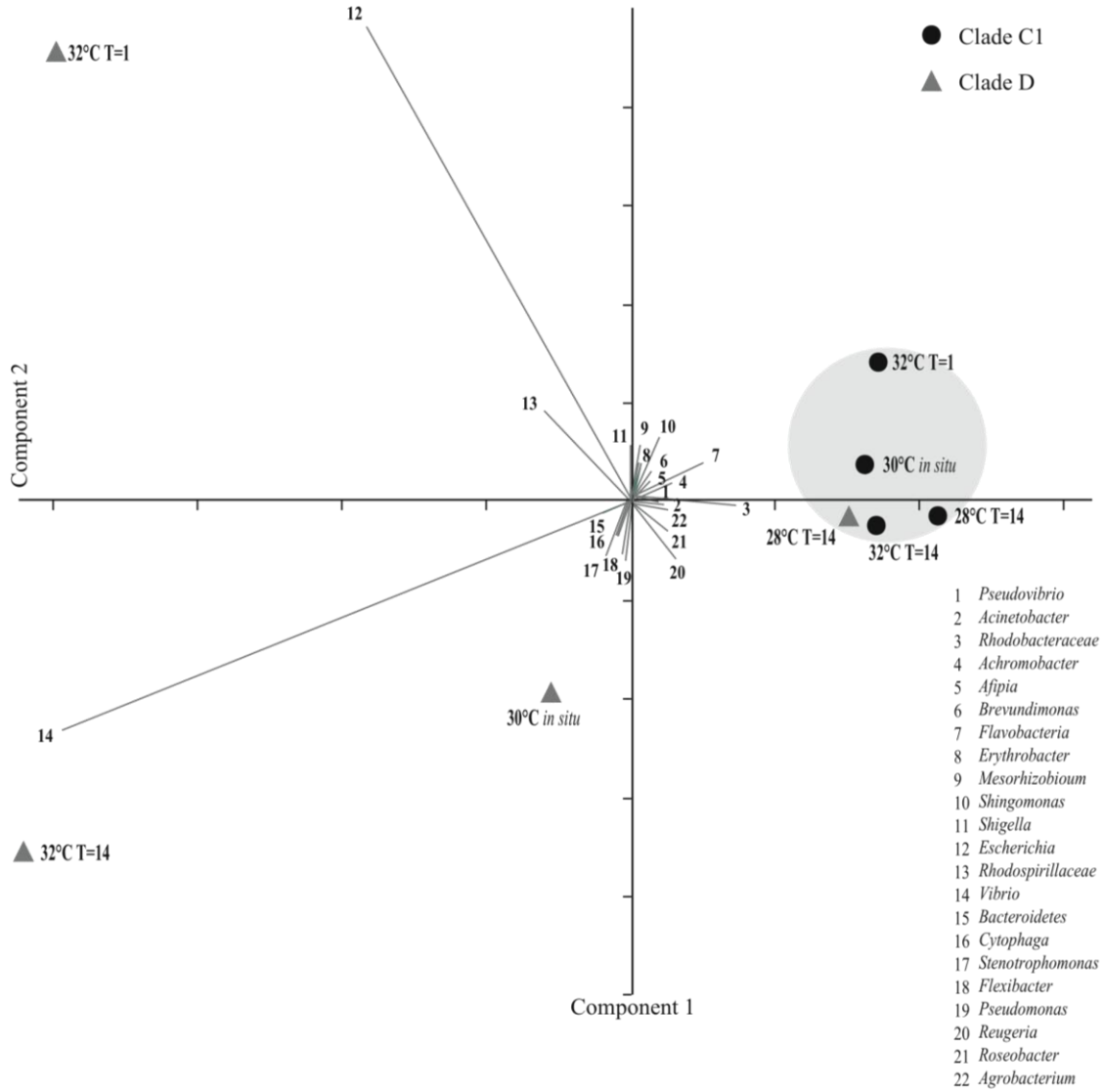

Figure 5.2 Principal Components Analysis (PCA) of clone libraries for corals hosting *Symbiodinium* ITS 1 type C1 or D at 28°C and 32°C for days 1, 12 and 14 of the experiment (i.e. T=1,12,14), as well as *in situ* samples (30°C). Analysis includes only dominant bacterial groups comprising 3% or greater of each clone library.

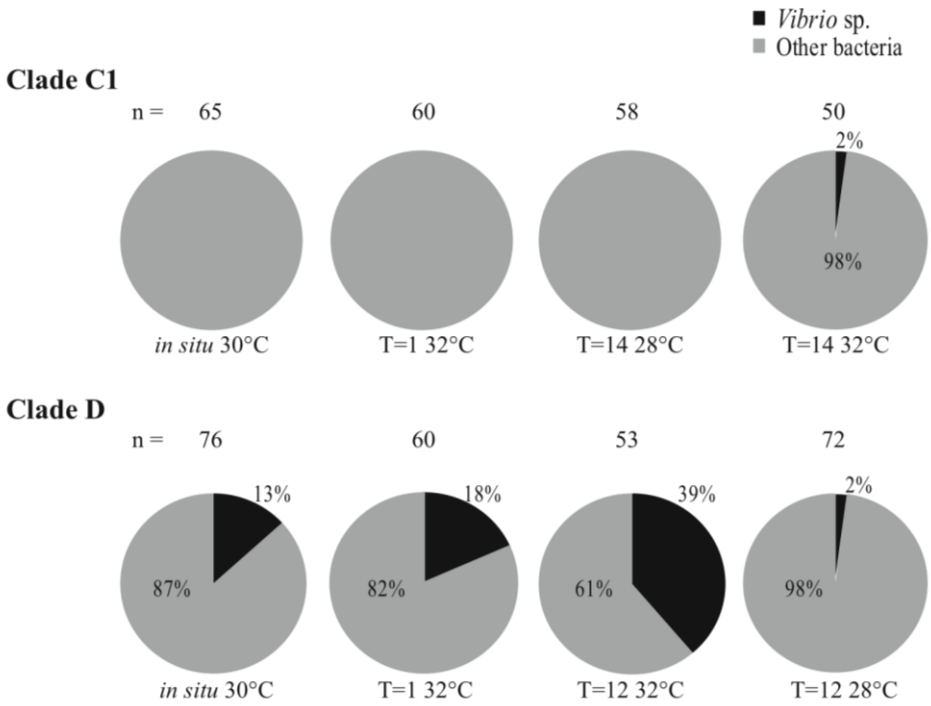

Figure 5.3 Proportion of *Vibrio* spp. affiliated sequences in clone libraries for *A. tenuis* hosting *Symbiodinium* ITS 1 type C1 or D at 28°C and 32°C for days 1, 12 and 14 of the experiment (i.e. T=1,12,14), as well as *in situ* samples (30°C). n= number of clone sequences.

5.3.3 Denaturing gradient gel electrophoresis (DGGE) profiling

Bacterial community profiles assessed by denaturing gradient gel electrophoresis (DGGE) displayed variable and complex banding patterns for replicate samples of both C1- and D-juveniles. These complex banding patterns again highlight the high diversity of coral bacterial associations. Nevertheless, some consistent groupings of bacterial DGGE banding patterns were observed when transformed into a non-metric multidimensional scaling plot. In particular, C1 and D-juveniles separated early in the experimental period (*in situ* and Days 1 to Day 5; Figure 5.4). The bacterial profiles of D-juveniles in the control treatment also cluster with profiles of C1-juvenile samples (Figure 5.4). This is consistent with the results of the clone library analysis, which similarly showed a shift in the associated bacterial profiles of these samples to

profiles closer to C1-juveniles in the absence of temperature stress. Although bacterial profiles of D-juveniles sampled from the 32°C treatment clustered in the nMDS for the first 5 days of the experiment, by day 9 (T=9), a shift in the coral-associated bacterial community occurred for these temperature stressed D-juveniles and by day 12, profiles differed from both the C1- and D-juveniles of the earlier sampled time points (Figure 5.4). Although bacterial profiles of C1-juveniles showed some drift in associated communities at the later time points in the experiment for both the 28°C and 32°C treatments, these were not consistent between replicate samples from the 28°C treatment and could be the result of aquarium effects.

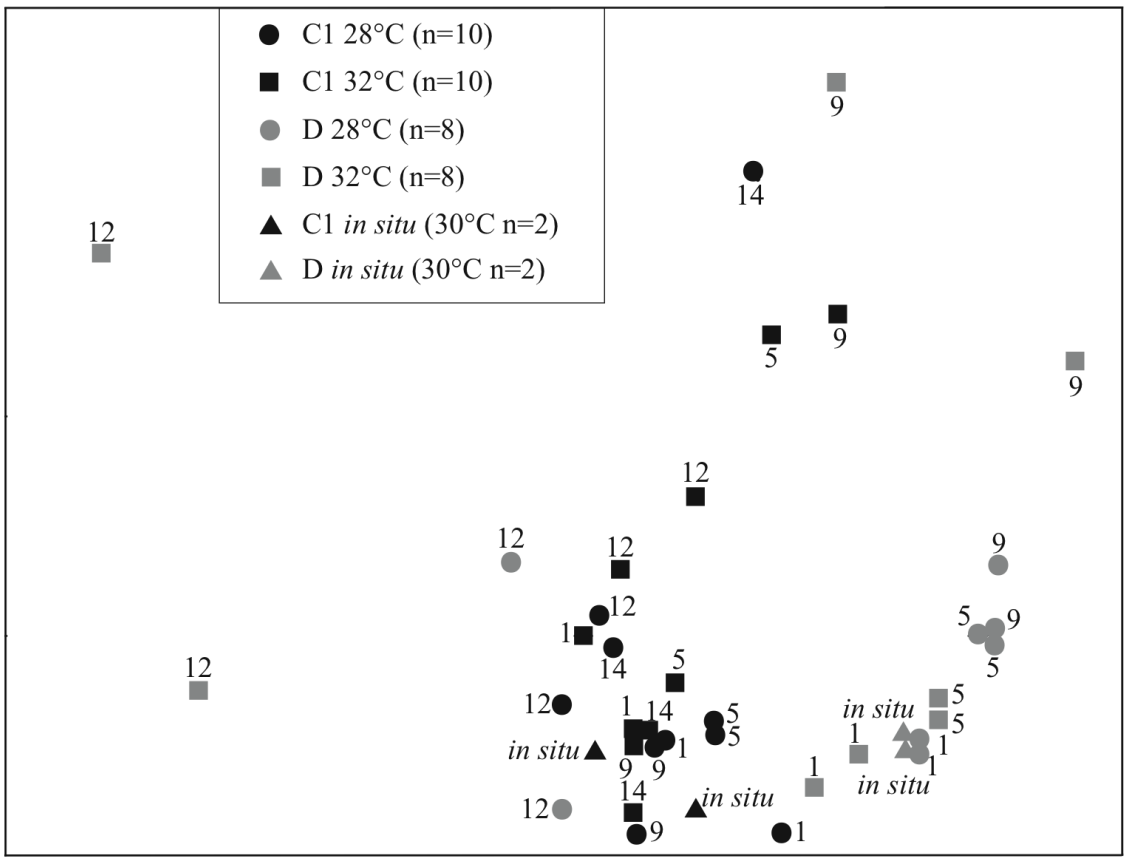

Figure 5.4 Non-metric Multidimensional Scaling (nMDS) of Denaturing Gradient Gel Electrophoresis (DGGE) profiles for *A. tenuis* hosting *Symbiodinium* ITS 1 type C1 or D at 28°C, 32°C and 30°C *(in situ* sample). Profiles are for *in situ* samples and samples collected on days 1, 5, 9, 12 and 14 (for C1 only) of experiment (i.e. T=1,5,9,12,14). Relative distance between points indicates level of similarity of DGGE profile.

Sequences recovered from replicate DGGE sample profiles were affiliated with *Brevundimonas* sp., *Stenotrophomonas* sp., *Muricauda* sp., *Achromobacter* sp. and *Ruegeria* sp. (Table 1, Figure 5.5) and thus corresponded to sequences retrieved from clone library analyses. Interestingly, one distinct DGGE band (Band 9) was consistently observed in the profiles of D-juveniles. The retrieved sequence of band 9 affiliated with *Vibrio* sp. related sequences, which was also consistent with results from the clone libraries; i.e. that D-juveniles possessed a higher relative proportion of *Vibrio* sp. sequences. Although profiles generated from C1-juveniles occasionally also possessed this band, the proportion of DGGE profiles with the *Vibrio* sp. band was much higher in samples of D-juveniles (Figure 5.6). All D-juvenile samples from the 32°C treatment and 80% of those from the 28°C treatment contained *Vibrio* sp. affiliated sequences (Figure 5.5). In contrast, only 16% of DGGE profiles for C1-juvenile samples from the 28°C treatment contained *Vibrio* sp. sequences. A slight increase in the appearance of *Vibrio* sp. sequences was observed in clade C1-juvenile samples in the elevated temperature treatment, with 29% of DGGE profiles containing the *Vibrio* sp. band (Figure 5.6).

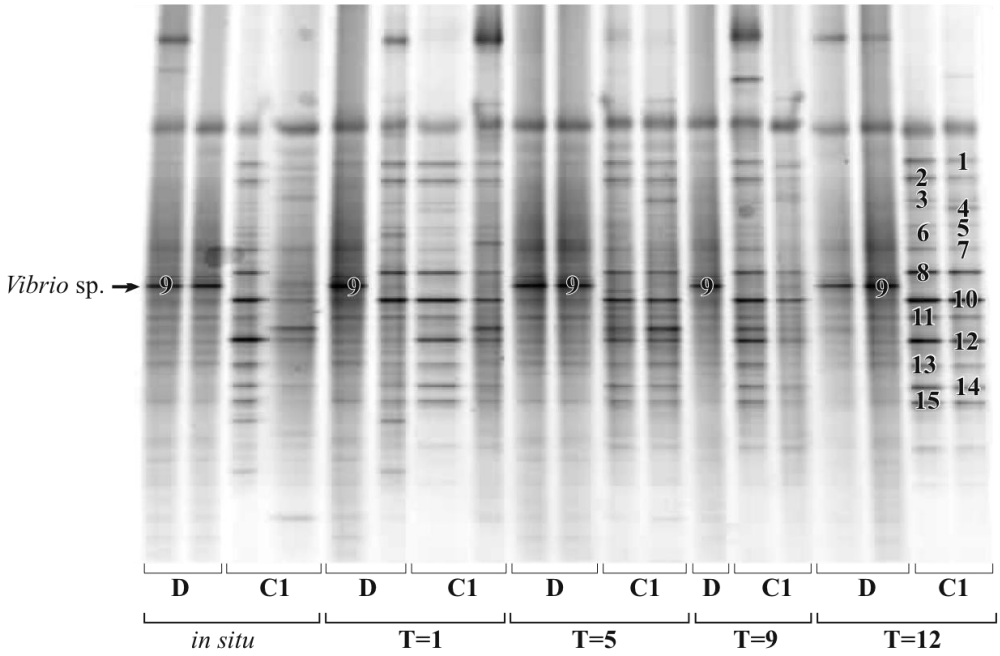

Figure 5.5 Example of Denaturing Gradient Gel Electrophoresis (DGGE) profiles for juveniles from the 32°C treatment for samples collected *in situ* and on days 1-12. T=day of the experiment. Numbers correspond to band affiliation in Table 1. C1 and D denote *Symbiodinium* ITS 1 type harboured. One T=9 replicate is missing because of low amounts of sample DNA.

Table 5.1 Affiliation of DGGE bacterial sequences retrieved from heat stressed juvenile coral colonies (n=38)

Band	Closest relative and database accession number	Alignment (bp)	Similarity* (%)	Taxonomic description
1	Uncultured bacterium (DQ684483)	252/310	81	*γ -Proteobacteria*
2	Uncultured bacterium (EU808101)	292/294	99	*α-Proteobacteria*
3	*Anoxybacillus* sp. (EU926955)	278/282	98	*Firmicutes*
4	*Sphingobium* sp. (EU679660)	270/295	91	*α-Proteobacteria*
5	*Acidovorax* sp. (EU639061)	254/255	99	*β-Proteobacteria*
6	*Nautella italica* (AM944522)	269/273	98	Unclassified *Proteobacteria*
7	Uncultured bacterium (EU535510)	270/309	87	*β-Proteobacteria*
8	*Achromobacter xylosoxidans* (EU877076)	283/285	99	*β-Proteobacteria*
9	*Vibrio* sp. (EU372935)	307/311	98	*γ -Proteobacteria*
10	*Alcaligenes* sp. (EU304282)	297/299	99	*β-Proteobacteria*

11	*Brevundimonas* sp. (AB447548)	265/269	98	*α-Proteobacteria*
12	Uncultured bacterium (EU010191)	287/315	91	Unclassified
13	*Flavobacterium frigidarium* (AY771738)	304/309	98	*Bacteroidetes*
14	*Rubritalea tangerina* (AB297806)	277/293	94	*Verrucomicrobia*
15	*Roseovarius sp.* (EU603447)	269/275	97	*α-Proteobacteria*

* Sequences were aligned to the closest relative using BLAST (Altschul, 1997). The similarity was calculated with gaps not taken into account.

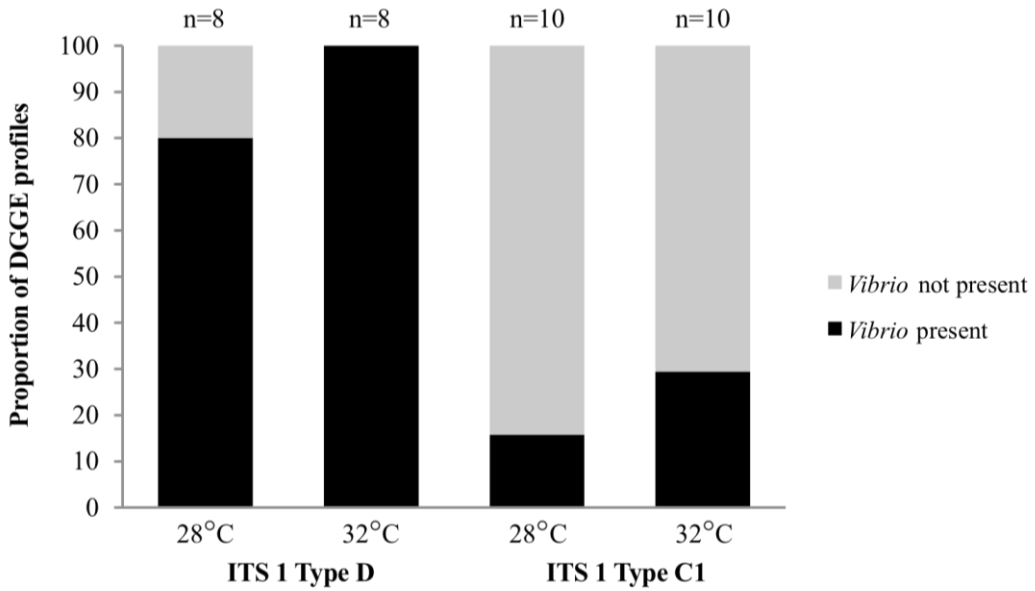

Figure 5.6 Proportion of Denaturing Gradient Gel Electrophoresis (DGGE) profiles containing *Vibrio* spp. affiliated sequences. n= number of total DGGE profiles analysed for each *Symbiodinium* type.

5.4 Discussion

This study demonstrates that the responses of coral-associated bacterial communities exposed to heat stress can differ for juvenile corals hosting different *Symbiodinium* partners. For example, under heat stress we found minimal changes in bacterial communities associated with juveniles of the coral, *Acropora tenuis*, when it

hosted *Symbiodinium* C1. In contrast, distinct shifts in bacterial communities on *A. tenuis* juveniles hosting *Symbiodinium* D were observed when exposed to the same elevated temperature treatment. Approximate halving in the photochemical efficiency of D-juveniles (44% reduction in F_v/F_m) combined with their white appearance provided corroborative evidence that D-juveniles were heat stressed. In contrast, C1-juveniles sampled from the elevated treatment after 14 days showed only a 10% reduction in F_v/F_m and most were only pale, indicating that, although under some heat stress, it was far less severe than that experienced by D-juveniles. Differences in the extent of change in bacterial community structure between the D- and C1-juveniles of *A. tenuis* when exposed to heat stress highlight the complexity of interactions that occur among microbial associates of corals, which, in combination, determine the health of the coral holobiont. Shifts in bacterial communities associated with temperature stressed D-juveniles also underscore the dynamic nature of microbial associations comprising the coral holobiont, as well as the potential for coral-associated microbial communities to respond to changes in environmental parameters.

Under persistent elevated temperatures, bacterial associations of D-juveniles appeared to break down, which coincided with the proliferation of sequences affiliated with *Vibrio* sp. For example, a three-fold increase in the proportion of *Vibrio* spp. sequences retrieved from clone libraries of D-juveniles in the elevated (compared to the control) temperature treatments was observed (Figure 5.3). Sequences related to *Vibrio* sp. were also retrieved from 100% of DGGE profiles of D-juveniles (Figure 5.6). These results are consistent with correlations found between the presence of *Vibrio* sp. and macroscopic signs of bleaching in molecular profiling studies of bacterial communities associated with corals undergoing a natural bleaching event (Bourne et al., 2008). Representation of the DGGE banding patterns within a nMDS

plot also clearly show a breakdown in bacterial associations, with D-juveniles exposed to 32°C and sampled at T=9 and T=12 days having variable bacterial profiles quite different from other sampling times (Figure 5.4). Similar bacterial shifts were observed in the sponge *Rhopaloeides odorabile* subjected to elevated temperatures. Webster et al. (2008) found that symbiotic bacteria were completely lost when water temperatures reached the sponge's upper thermal threshold, which coincided with the establishment of 'alien' bacterial species. In contrast, juveniles harbouring *Symbiodinium* C1 did not exhibit the same loss or shift in bacterial associates. For example, the proportion of *Vibrio* sp. related sequences increased only marginally to 2% of the clone library for C1-juveniles held at 32°C (Figure 5.3). DGGE profiles of juveniles sampled from the elevated temperature treatment also showed dramatically fewer *Vibrio* sp. bands retrieved for C1-juveniles (<33% of profiles) in comparison to D-juveniles (Figure 5.6). Additionally, clustering of the dominant bacterial sequences retrieved for C1-juveniles in the 28°C treatment with those retrieved for C1-juveniles in the 32°C treatment, in both the PCA (Figure 5.2) and nMDS DGGE (Figure 5.4) profile representations, provides further evidence that the bacterial communities of C1-juveniles were not as disrupted as those of D-juveniles.

Retrieval of *Vibrio*-affiliated sequences from D-juveniles sampled in the field before the start of the experiment suggests that these juveniles may have been stressed prior to experimentation. The moderately high seawater temperatures of ~30°C, which is towards the upper end of the thermal tolerance range of *Acropora* species (Berkelmans and Willis 1999), recorded at the field site prior to the start of the study supports this interpretation. Moreover, the reduced number of *Vibrio* sequences retrieved from D-juveniles in the 28°C (control) treatment (Figure 5.3) provides further corroborative evidence that these juveniles were stressed at the 30°C temperatures

experienced in the field. Consistency in results of the PCA analysis of clone libraries and the nMDS analysis of DGGE banding patterns, both of which demonstrated that profiles of D- and C1-juveniles maintained at 28°C clustered together, primarily as a consequence of D-juveniles shifting their bacterial profiles to become more similar to profiles of C1-juveniles (Figure 5.4; *in situ* and T=12 at 28°C), adds further weight to this interpretation. Therefore, it is likely that D-juveniles were under heat stress *in situ* and the removal of this stress when placed into the control treatment (28°C) for two weeks resulted in a decrease of *Vibrio* sp. associated with these samples. However, sampling of coral-associated bacteria at different times of year would be required to confirm this conclusion.

Members of the *Vibrionacae* family have been linked with an array of coral diseases. For example, *Vibrio coralliilyticus* has been shown to be a causative agent for some types of Indo-Pacific white syndromes (Sussman et al. 2008), as well as the aetiological agent for bleaching and tissue lysis in *Pocillopora damicornis* (Ben-Haim and Rosenberg 2003). Four strains of *Vibrio* sp. were also found to induce signs of yellow blotch disease on *Montastraea* spp. in the Caribbean (Cervino et al. 2004), and *Vibrio shiloi* has been established as the causative agent of coral bleaching in the Mediterranean (Kushmaro et al. 1996, Kushmaro et al. 2001). An increase in abundance of other *Vibrio* spp. has also been shown to coincide with other coral diseases (Barneah et al. 2007, Arotsker et al 2009, Piskorska et al 2007, Breitbart et al. 2005) and environmental stress (Bourne et al. 2008, Vega-Thurber et al 2009). Disease causation is a complex interaction between the host, causative agents and the environment (Work et al. 2008; Bourne et al., 2009). In some cases, correlation between the emergence of a coral disease and the appearance of *Vibrio* spp. indicates that members of this family may be primary pathogens that cause infections. However,

in other cases, *Vibrio* spp. residing on the surface of corals may proliferate when factors, such as elevated temperatures change the homeostasis of coral microbial associations through lowering host resistance. In our study, correlation between heat stress and *Vibrio* dominance indicates that *Vibrio* species may be either primary pathogens or alternatively opportunistically taking advantage of a compromised host. In either case, their presence partially explains the high mortality rates of D-juveniles in the field before they were placed into experimental conditions.

The differential susceptibility of corals harbouring different *Symbiodinium* types to *Vibrio* proliferation, as observed in the current study, is consistent with previous studies of disease resistance in corals harbouring different *Symbiodinium* types. For instance, Sussman et al. (2009) found that *A. millepora* may be more susceptible to PSII inactivation by *Vibrio* zinc-metalloprotease when hosting *Symbiodinium* type A. However, Sussman et al. (2009) found corals harbouring ITS 1 types C1 and D had similar susceptibilities to photo-inactivation, suggesting that this mechanism does not explain differences observed between C1- and D-juveniles in our study. Stat et al. (2008) found sub-optimal health states of *Acropora cytherea* harboring clade A, as well as increased incidence of disease compared with corals harbouring clade C. Moreover, significantly higher amounts of carbon were found to be released by clade C than clade A, suggesting that differences in nutrient acquisition could have contributed to differences in disease susceptibility. In contrast, Correa et al (2009) found that specific *Symbiodinium* types were not correlated with diseased tissues when comparing healthy and diseased tissue from the same coral. However, it is possible that sub-optimal partnerships with *Symbiodinium* could enhance vulnerability to opportunistic infections.

Use of coral juveniles for our experimental treatments enabled manipulation of the *Symbiodinium* type *in hospite* and minimized the likelihood of changes in bacterial communities as a consequence of physical stress associated with fragmentation. Thus, only small shifts in bacterial associates were observed on control juveniles throughout our study, including minimal changes between juveniles in the field and in control treatments, and at the beginning and end of the experiment. However, the microbial associates of juvenile corals differ markedly from those of adult corals (Littman et al. 2009b). Highly diverse coral bacterial associations were found associated with juveniles in this study (Table 1), similar to those found in a previous investigation (Littman et al. 2009b), providing further evidence that bacterial associates are not conserved in the early stages of coral ontogeny. The appearance of more structured bacterial communities after one year (Littman et al. 2009b) suggests that a winnowing process (*sensu* Nyholm and McFall-Ngai 2004) potentially takes place to establish diverse yet conserved bacterial symbioses within adult corals (Rohwer et al 2002; Littman 2009a). Further studies of interactions among bacterial associates, *Symbiodinium* type and the coral host once stable adult associations are established would provide further insights into processes underpinning the thermal tolerance and health of corals.

In conclusion, the stability of coral-associated bacterial communities appears to be correlated to the ITS 1 *Symbiodinium* type harboured by the coral host. Moreover, *A. tenuis* hosting *Symbiodinium* D became dominated by *Vibrio* species under elevated temperature stress and showed signs of declining health, whereas juveniles hosting *Symbiodinium* C1 experienced only minor shifts in bacterial community structure. Although most previous studies have suggested that corals associated with *Symbiodinium* D typically have high tolerance to thermal stress (Glynn et al. 2001;

Toller et al. 2001; Baker et al. 2004; Fabricius et al. 2004; Rowan 2004; Berkelmans and van Oppen 2006), our results concur with those of Abrego et al. (2009), who showed that C1 is the optimal *Symbiodinium* type for *A. tenuis* under thermal stress. In combination, these studies highlight that associating with *Symbiodinium* type D does not necessarily confer the same physiological response in different coral species. If corals switch or shuffle their *Symbiodinium* types in response to temperature stress, as proposed by the adaptive bleaching hypothesis (Buddemeier and Fautin 1993; Baker 2003), this may result in corals having different susceptibilities to opportunistic pathogens. Thus, although changing *Symbiodinium* types following bleaching events may provide immediate benefits to the coral holobiont in terms of thermal tolerance, it could also result in a longer-term trade-off with disease resistance. These studies demonstrate the necessity for a holistic approach in order to understand the complexity of inter-kingdom interactions that together govern the health of the coral holobiont.

Chapter 6.0 Metagenomic analysis of the coral holobiont during a natural bleaching event on the Great Barrier Reef

This chapter is inserted without abstract as published in the journal *Environmental Microbiology Reports*:

Littman, R. A., Willis, B. L., Bourne, D. G. (2011) Metagenomic analysis of the coral holobiont during a natural bleaching event on the Great Barrier Reef. *Environmental Microbiology Reports*. (in press).

All the data was collected and analyzed by R. Littman, who also wrote the chapter and manuscript after intellectual contributions by all co-authors.

6.1 Introduction

Corals form close associations with a complex consortium of microorganisms, including *Symbiodinium* (zooxanthellae), *Fungi*, endolithic algae, *Bacteria*, *Archaea* and viruses, with the collective group of organisms referred to as the coral holobiont (Rohwer et al., 2002; Reshef et al., 2006). *Symbiodinium* associates are well known for their role as a keystone symbiotic partner of the coral, supplying up to 95% of the host's energy requirements and enhancing coral growth (Muscatine, 1973). However, much less is known about the composition and functional roles of other microbial associates that populate internal and external layers of the coral host.

Knowledge of the diversity and function of marine microbiota has rapidly expanded in recent decades through the emergence of molecular techniques that target relevant phylogentic markers and functional genes. Recent development of pyrosequencing approaches to metagenomics has greatly expanded our capacity to answer complex environmental questions by enabling analysis of the near-complete collection of genes within identified niches without isolation and cultivation of individual microbial components. This approach provides a description of the taxonomic constituents of environmental samples (Tyson et al., 2004), the relative abundances of taxa and genes (Breitbart et al., 2002, Angly et al., 2006) and identification of many genes involved in biosynthetic and metabolic pathways (Shirmer et al., 2005; Tringe et al., 2005; Rodriguez-Brito et al., 2006). In combination, this information enables assessment of the functional characteristics of complex microbial communities.

Recently, metagenomics has been applied to investigations of taxonomic diversity, as well as metabolic capabilities of microbial members of the coral

holobiont. Wegley et al. (2007) investigated the possible roles of each component of the microbial community associated with the coral *Porites astreoides* and proposed new models for nutrient cycling within the coral using the inferred genetic capabilities of the microbial members. Vega-Thurber et al. (2009) further explored shifts in taxa and function of the microbiota associated with *Porites compressa* under different environmental stressors (temperature, dissolved organic carbon, pH and nutrients) and highlighted increases in the relative abundance of stress resistance and virulence genes in stressed corals, in addition to changes in community metabolism that differed according to the stress applied on the coral host. Detailed analysis of the controlled heat stress treatment showed that metabolic changes in *Vibrio* spp., even when in low abundance, led to major shifts in the entire coral microbiome, and also demonstrated shifts from a healthy coral-associated community to microbes associated with disease. While highly informative, this study had the caveat that treatments did not reflect realistic stressors because a single extreme stress was applied over a short time span with pseudo-replication.

Coral reefs have been identified as one of the most vulnerable ecosystems on the planet (Wilkinson, 2008). Increased frequency and intensity of coral bleaching events are predicted in the future as a consequence of warming oceans driven by climate change, and will inevitably cause loss of reef habitat and biodiversity (Brown, 1997; Hoegh-Guldberg et al., 2007). Because of the coral microbiota play important roles in nutrient cycling and antimicrobial protection for the coral holobiont (Ritchie and Smith, 2004; Rohwer and Kelley, 2004; Reshef et al., 2006), it is important to investigate the effects of elevated temperature and bleaching on the functional roles of coral microbial communities. Accordingly, we employed metagenomic techniques to analyse samples of *Acropora millepora* collected before and during a natural bleaching

event on the Great Barrier Reef in 2002 (Berkelmans et al., 2004) to gain further insight into metabolic and taxonomic shifts that occur in coral-associated microbial communities during a natural thermal bleaching event.

6.2 Materials and methods

6.2.1 Sampling of Acropora millepora

Colonies of the coral *Acropora millepora* were tagged on the reef flat (1.5 - 3 m depth) of Nelly Bay, Magnetic Island (19º10's 146º50'E) and sampled repeatedly over three years, which included the major 2001/2002 bleaching event on the Great Barrier Reef (Berkelmans et al., 2004; samples stored at -80°C until analysis; see Bourne et. al., 2008 for further details). Samples from five colonies collected on 31st October 2001 were termed "healthy' and samples from the same five colonies collected during the peak of the thermal stress event (7th February 2002) were termed 'bleached". All five colonies subsequently recovered after being bleached for approximately 4 weeks.

6.2.2 DNA extraction and amplification

Samples were crushed and homogenized as described in Wegley et al. (2007). Coral-derived microbial cells were separated from coral nuclei and zooxanthellae using centrifugation and Percoll fractionation (see Wegley et al., 2007). DNA was extracted using a MO BIO PowerPlant® DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA). Total Genomic DNA was amplified from each of the five replicate sample using GenomiPhi (Amersham Biosciences, Pittsburgh, PA), subsequently pooled to produce one healthy and one bleached library, before sequencing using 454 Life Sciences pyrosequencing and titanium chemistry.

6.2.3 Bioinformatics

The total number of reads was 395,998 for the healthy library and 395,627 for the bleached library, with an average sequence length of 330 base pairs. The number of reads is comparable to sequence results reported in Wegley et al. (2007) of 316,279 reads, although with longer average read length. Sequences were compared to the SEED non-redundant database, which uses BLASTX (Overbeek et al., 2004), and E-value cutoffs were set at 10^{-5} with a minimum alignment length of 50bp. The MG-RAST server was used to classify genes into general metabolic categories (systems), which are also divided further into metabolic subsystems. Statistical analyses to determine differences in metabolic and taxonomic systems and subsystems between the healthy and bleached metagenomes were performed using STAMP (Parks and Beiko, 2010). Corrected p-values were calculated using Storey's FDR approach (95% confidence intervals). To account for variability in the number of sequences between the healthy and bleached libraries, the proportion of genes within each of the 23 metabolic systems (see Figure 1) was calculated, by dividing the number of sequences associated with each metabolic system by the total number of sequences classified in each library.

6.3 Results

6.3.1 Shifts in metabolic systems

Sequences attributed to photosynthesis and respiration together comprised more than 85% of the healthy library (Figure 6.1), but both categories were reduced by more than 30% in the bleached library. For the healthy coral, 48% of classified sequences were assigned as photosynthetic genes and 39% were respiratory genes, dropping to 30% and 23% of classified sequences for photosynthesis and respiration, respectively, in the bleached library (Figure 6.1a). Concurrently, carbohydrate, sulfur, phosphorous

and fatty acid metabolism increased by two to six-fold in the bleached library (Figure 6.1b).

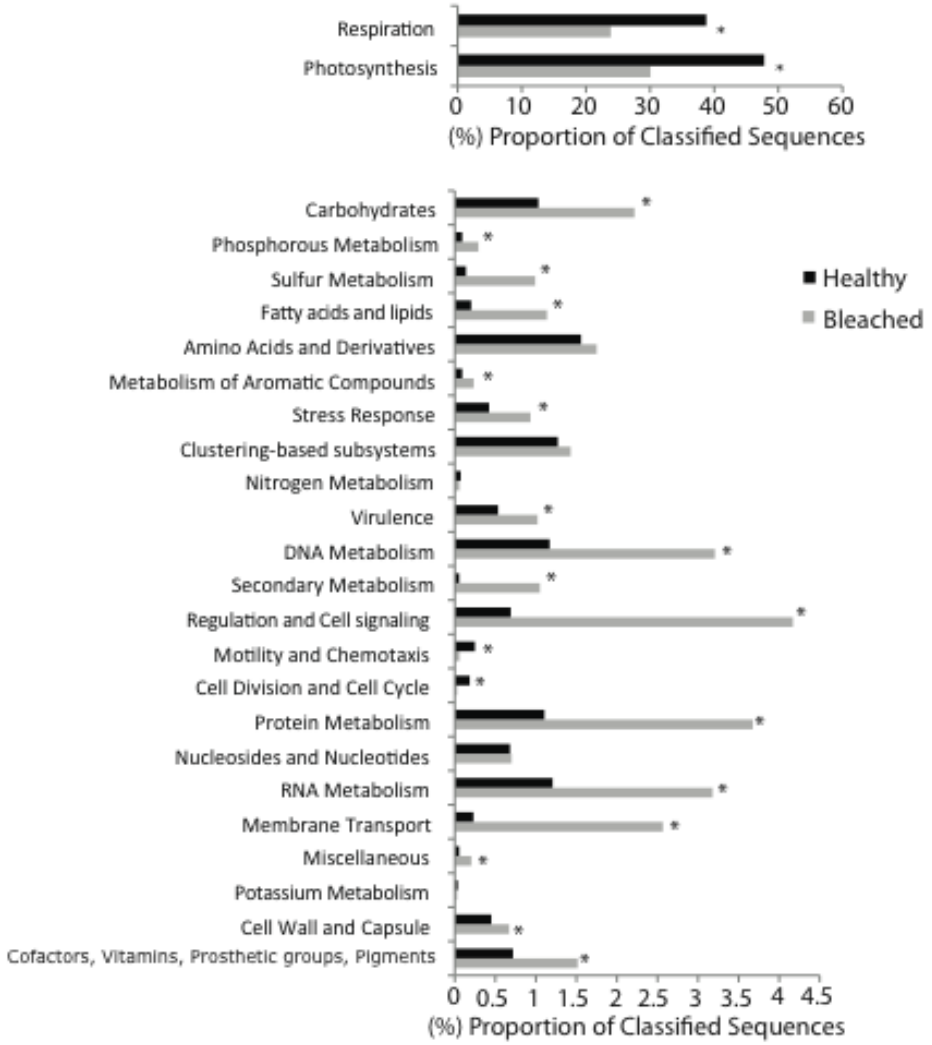

Figure 6.1 Proportion of sequences classified into metabolic systems. Asterisks indicate statistically significant differences between the bleached and healthy systems as determined by STAMP.

To investigate shifts in nutrient assimilation and both anabolic and catabolic processes, subsystems of genes within the sulfur, nitrogen, phosphorous, carbohydrate, protein and fatty-acid metabolic categories were further examined to determine which subsystems were responsible for shifts observed at the system level (Figure 6.2). For carbohydrates, 18-fold increases in sugar alcohol and 4-fold increases in di- and oligosaccharide, were detected in the bleached library, representing 0.17% and 0.84%,

respectively, of the bleached library. Phosphate metabolism significantly increased in the bleached metagenome by nearly 2-fold, representing 0.26% of the bleached library. Increases in sulfur metabolism were attributed to the 9-fold increase in Galactosylceramide and Sulfatide metabolism (to 0.99%) in the bleached metagenome.

Both protein biosynthesis and degradation increased by 2 to 10-fold in the bleached library (to 1.72% and 1.48%) but no significant differences in nitrogen metabolism were found (Figure 6.2). All three subsystems of fatty acid metabolism were elevated by 2 to 21-fold in the bleached library, with phospholipids, triacyglycerols and fatty acids reaching 0.32%, .20% and 0.49% of the library (Figure 6.2). In addition, there were marked elevations in DNA metabolism (>2-fold increase to 3.20%), regulation and cell signaling (>5-fold increase to 4.17%), protein metabolism (>2-fold increase to 3.67%), RNA metabolism (2-fold increase to 3.18%) and membrane transport (>10-fold increase to 2.56%) (Figure 6.1).

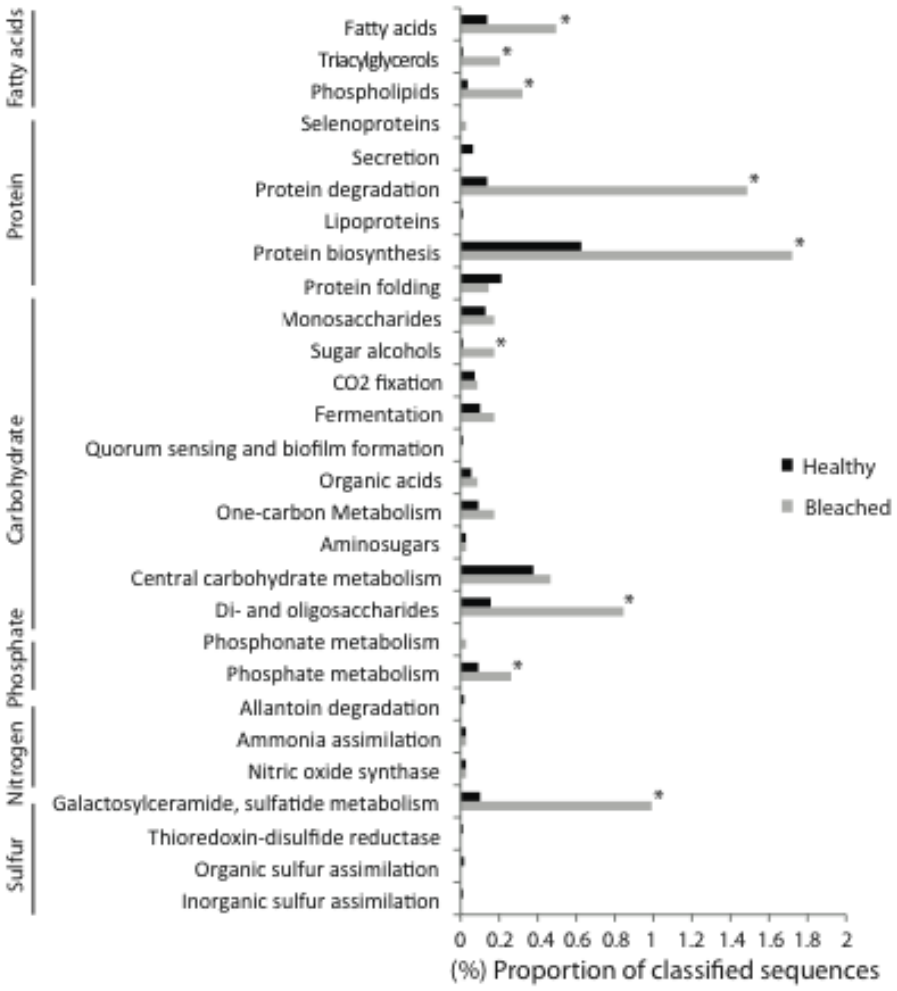

Figure 6.2 Proportions of sequences classified into nutrient acquisition subsystems. Asterisks indicate statistically significant differences between the bleached and healthy subsystems as determined by STAMP.

6.3.2 Bleaching leads to increases in stress and virulence genes

Of the other metabolic categories that increased significantly within the bleached coral metagenome (Figure 6.1), it is notable that genes associated with secondary metabolism increased more than eighteen-fold and those associated with stress responses and virulence increased by approximately two-fold. Accordingly, stress, virulence and secondary metabolism genes were also examined at the subsystem level

to determine which gene categories have implications for coral health following thermal stress (Figure 6.3). For Secondary metabolism, the most notable gene category was biosynthesis of phenylpropanoids, which increased by more than twelve-fold (to 0.61%) in the bleached coral metagenome. Phenylpropanoids encompass a variety of compounds produced by plants when they are exposed to biotic or abiotic stressors, such as wounding, pathogenic attack or UV radiation (Dixon and Paiva, 1995). Similarly, genes associated with osmotic and oxidative stress, along with genes assigned to DNA repair were all 1 to 2-fold higher (to 0.17%, 0.47% and 2.71%) in the bleached metagenome. The largest change observed in subsystems within the virulence category was a seven-fold increase (to 3.70%) in sequences assigned to proteolytic pathways (Figure 6.3).

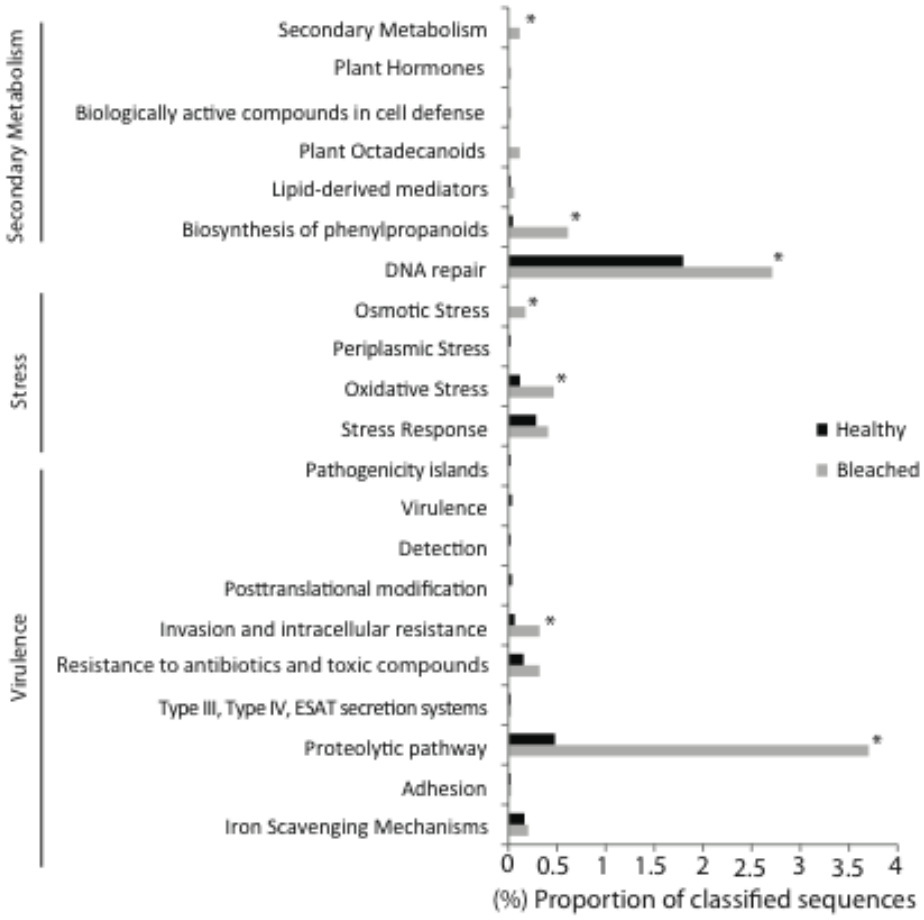

Figure 6.3 Proportions of sequences classified as stress response subsystems. Asterisks indicate statistically significant differences between the bleached and healthy subsystems as determined by STAMP.

6.3.3 Microbial taxonomic shifts

Of the total number of sequences retrieved, only 6% (i.e. 23,507 sequences) in the healthy library and 5.5% (i.e. 22,009 sequences) in the bleached library could be classified and assigned to taxonomic categories. Micro-algae comprised the largest component of retrieved microbial genes from both libraries, representing 28% of healthy and 6% of bleached sequences classified (i.e. 6590 and 1322 sequences, respectively). Fewer sequences were assigned to the *Bacteria*, with bacterial sequences making up 15.3% (i.e. 3599 sequences) of those classified in the healthy

library, but only 4.3% (947 sequences) of those classified in the bleached library. In contrast, more virus-like sequences were retrieved from the bleached library (2.8%, 609 sequences) than the healthy library (0.8%, 185 sequences). *Archaea* and *Fungi*-like sequences were retrieved from both metagenomes, with 0.39% and 0.40% (95 and 87 sequences, respectively) of healthy and bleached sequences assigned as *Fungi*, and 0.20% (43 sequences) and 0.10% (27 sequences) classified as *Archaea* for healthy and bleached libraries, respectively (Figure 6.4). By far, the largest component of both libraries were classifies as Metazoa and likely derived from mitochondria which are a similar size to bacteria and carried through in the microbial fractionation.

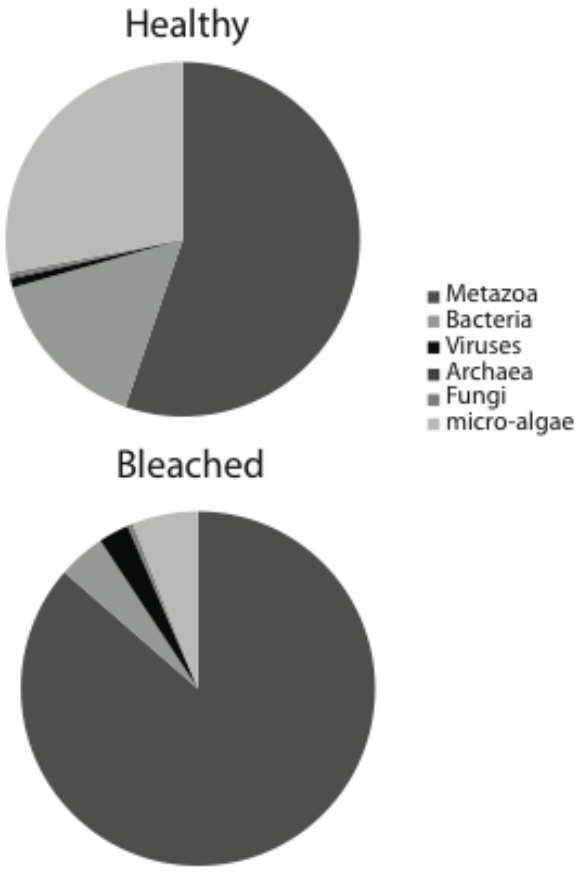

Figure 6.4 Overview of the taxonomic classifications of sequences in healthy and bleached metagenomes.

The dominant bacteria in the healthy library were the *Cyanobacteria* (1552 sequences), followed by the *Proteobacteria* (781 sequences), *Firmicutes* (596 sequences), *Actinobacteria* (250 sequences), *Bacteroidetes* (123 sequences) and *Chlorobi* (93 sequences). The only significant shift in bacterial groups detected between the healthy and bleached libraries was a 7% decrease in the proportion of *Firmicutes* in the bleached library (data not shown). When bacterial groups within each of these phyla were examined further, significant changes were detected between healthy and bleached libraries. Within the *Firmicutes*, proportions of bacteria decreased by more than two-fold in the bleached library for *Chlorobi* (to 0.84% of bacterial sequences) and *Clostridia* (to 3.38% of bacterial sequences) (Figure 6.5). The proportions of *γ-Proteobacteria* increased by 60% (to 11.61% of bacterial sequences), but decreased by 30% in the *α-Proteobacteria* (to 7.18% of bacterial sequences) (Figure 6.5). Of the cyanobacteria, *Chroococales* decreased by approximately 25% (to 20.27% of bacterial sequences), whereas the *Nostocales* and *Prochlorales* increased by approximately two-fold (to 15.10% and 1.90%, of bacterial sequences respectively) in the bleached library (Figure 6.5).

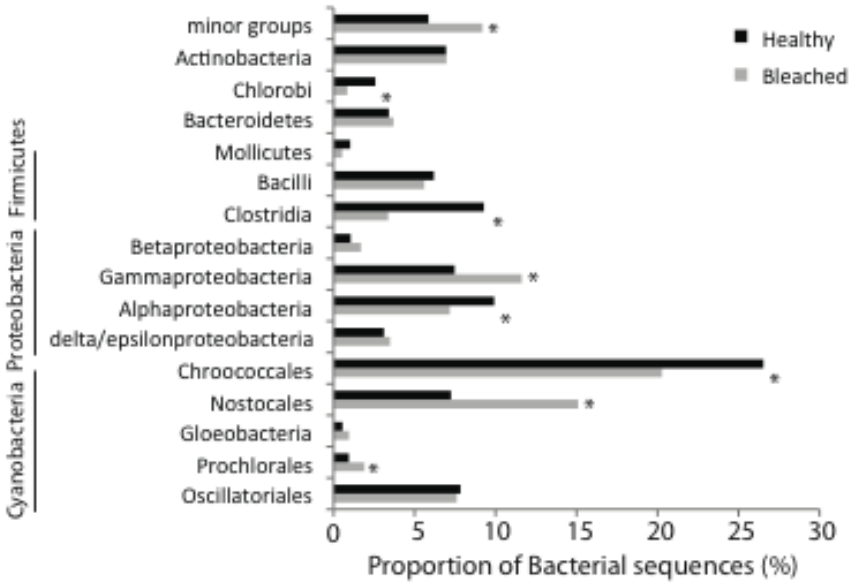

Figure 6.5 Changes in the proportions of sequences classified as Bacteria during bleaching. Asterisks indicate statistically significant differences between the bleached and healthy systems as determined by STAMP.

6.3.4 Bleaching causes different changes in the Eukaryotic community

Bleaching resulted in dramatic shifts in some members of the Eukaryotic community associated with corals (Figure 6.6). For example, *Fungi*-like sequences increased three-fold (to 6.58% of Eukaryotic sequences) in the bleached metagenome. Most other Eukaryotic sequences decreased in the bleached library, including 9% decreases in stramenopiles (to 22.69% of Eukaryotic sequences), 30% decrease in *Rhodophyta* (to 26.55% of Eukaryotic sequences) and two-fold decrease in *Cryptophyta* (to 6.2% of Eukaryotic sequences). Only a small proportion of sequences were assigned to the *Alveolata* (0.2% to healthy, 2.0% to bleached), which include the *Symbiodinium,* indicating that this group was successfully removed during the fractionation process. Interestingly, the proportion of sequences assigned to

Viridiplantae, which includes the endolithic algae, *Ostreobium*, increased by ~50% (to 27.38% of Eukaryotic sequences) in the bleached library.

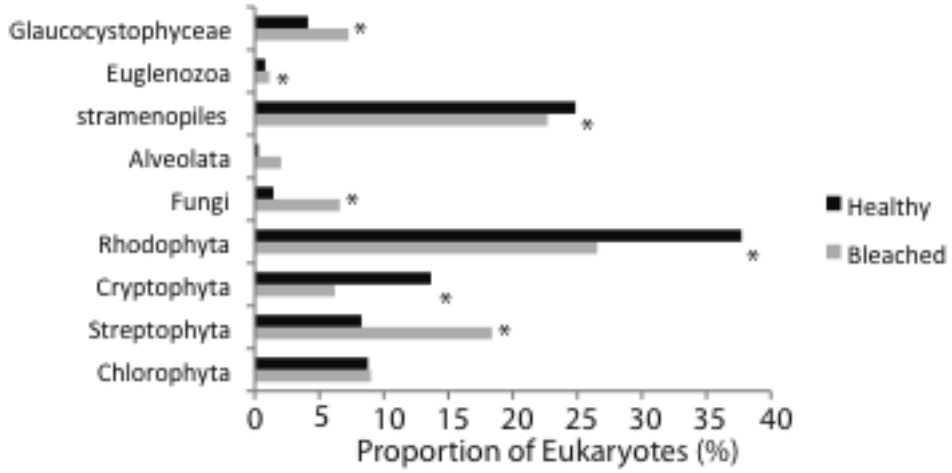

Figure 6.6 Changes in the proportions of sequences classified as Eukaryotes during bleaching. Asterisks indicate statistically significant differences between the bleached and healthy systems as determined by STAMP.

6.3.5 Low Archaeal sequence abundance

Very few *Archaeal* sequences were recovered from either the healthy or bleached metagenomes. Of the archaeal sequences retrieved, *Euryarchaeota* and *Crenarchaeota*-like sequences were the largest number recovered from the healthy and bleached metagenomes. Only two sequences were assigned to *Korarchaeota* for the healthy library (Table 6.1). *Crenarchaeota* sequences were significantly elevated by 40% in the bleached library.

Table 6.1 Number of sequences classified as *Archaea.*

Archaea	No. sequence similarities in library	
	Healthy	**Bleached**
Crenarchaeota		
Thermofilaceae	4	13
Thermoproteaceae	5	1
Nitrosopumilales	1	0
Euryarchaeota		
Halobacteriaceae	6	0
Methanobacteriaceae	4	0
Methanocorpusculaceae	0	1
Methanomicrobiaceae	0	1
Methanomicrobiales	1	0
Methanopyraceae	1	0
Methanosarcinaceae	9	6
Methanospirillaceae	0	4
Picrophilaceae	2	0
Thermococcaceae	5	1
Thermoplasmataceae	3	0
Korarchaeota		
Korarchaeum	2	0

6.3.6 Elevated temperature increases coral associated viruses

Sequences related to Virus sequences were highly diverse. However, phages were the most abundant viruses associated with *A. millepora,* most of which were classifed as *Microviridae* (Table 6.2). The proportion of *Microviridae* was significantly elevated (15%) in the bleached metagenome.

Table 6.2 Number of sequences classified as Viruses in bleached and healthy metagenomes.

Viruses	Healthy	Bleached	Examples of observed taxa
dsDNA viruses			
Myoviridae	20	1	Cyanophage P-SSM2
Podoviridae	2	1	Cyanophage P-SSP7
Mimiviridae	1	8	Acanthamoeba polyphaga mimivirus
Phycodnaviridae	0	1	Paramecium bursaria Chlorella virus
Poxviridae	3	13	Fowlpox virus
Baculoviridae	2	0	Cydia pomonella granulovirus
Siphoviridae	1	0	Enterobacteria phage T5
ssDNA viruses			
Circoviridae	15	69	Bovine circovirus
Geminiviridae	0	4	Squash leaf curl Yunnan virus
Microviridae	112	463	Chlamydia phage
Nanoviridae	28	46	Subterranean clover stunt virus
ssRNA viruses			
Tombusviridae	0	2	Melon necrotic spot virus
Other			
Satellites	1	0	Tomato yellow leaf curl

6.4 Discussion

6.4.1 Shifts from autotrophy to heterotrophy

The decrease in the proportion of genes associated with photosynthesis and respiration and the concurrent increase in sequences assigned as carbohydrate, sulfur, phosphorous and fatty acid metabolism in the bleached metagenome indicate that the coral microbiome changed from a system driven by autotrophy to one comprised predominately of heterotrophic modes of metabolism (Figure 6.1). Photosynthesis provides the primary source of carbon for corals and as much as 20-40% of daily net photosynthates are released as exudates in coral mucus (Davies, 1984, Crossland, 1987, Edmunds and Davies, 1989), which in turn is used as a substrate by many of the microbial associates (Brown and Bythell, 2005). Thus, deprived of autotrophically-

derived substrates during bleaching, the microbial community potentially shifts towards other forms of nutrient acquisition.

It is possible that the increased phosphate metabolism we detected in bleached samples of *A. millepora* resulted from bacterial utilization of the coral's lipid conjugate. In a bacterial profiling study of soft corals throughout a bleaching experiment, Burdett et al. (2010) revealed that bacterial communities switched from predominately *Pseudomonas* species to *Vibrio* species. Based on evidence from markers for phosphonoacetate hydrolase (*phnA*) genes, they proposed that pathogenic *Vibrio* sp. were capable of hydrolysing the phosphonolipids within the membrane of the coral itself during heat stress (Burdett et al., 2010).

6.4.2 Increases in virulence sequences

The largest change observed in subsystems within the virulence category was the increase in sequences assigned to proteolytic pathways (Figure 6.3). Proteolysis is the breakdown of proteins into simpler substances, such as peptides or amino acids, and performs various biological roles, including the activation of a function, a signal in a signaling pathway or digestion of proteins. Increases in pathways used for degradation of various compounds are consistent with the microbial community switching from an autotrophic to a heterotrophic state. However, as suggested by Ben-Haim et al. (2003b) and demonstrated by Sussman et al. (2009), an extracellular protease associated with the pathogen *Vibrio coralliilyticus* is the virulence factor for *Symbiodinium* lysis in corals at high temperatures. It is possible that other bacterial pathogens may use similar virulence factors in the infection of coral. Therefore, the increase in proteolytic genes during heat stress could also be due to proliferation of disease-causing organisms.

6.4.3 Shifts in coral associated bacteria

Previous studies have found similar shifts in coral associated bacteria as in this study. While Vega Thurber et al. (2009) similarly found a significant decrease in *Firmicutes* during heat stress, they also found significant shifts in *Proteobacteria*, *Cyanobacteria*, *Chlamydiae* and *Actinobacteria* which not observed in this study. In agreement with our results, Koren and Rosenberg (2007) detected increased *γ-Proteobacteria* sequences retrieved from 16S rDNA gene analysis of bleached *Oculina patagonica* colonies, specifically *Acinetobacter* spp. related sequences. However, in contrast to our results, Mouchka et al. (2010) observed higher relative abundances of *α-Proteobacteria* related sequences, although this was in diseased rather than bleached coral tissues.

Bacteria, particularly *Vibrio* species, have been implicated as the causative agent in some cases of coral bleaching (Ben-Haim et al., 2002; 2003a; Kushmaro et al., 1996; 1997), but we did not observe any measurable increases of bacteria within the *Vibrio* group in the bleached relative to the healthy samples (data not shown). *Vibrio splendidus*, *V. harveyi* and *V. angustum* were recovered from both healthy and bleached samples. Similarly, Vega-Thurber et al. (2009) did not find an increase in *Vibrio* species in experimentally bleached samples, but did observe a significant increase in virulence genes from the *Vibrio* family, indicating this group contributed disproportionally to the coral holobiont biome under heat stress. While no increases in *Vibrio* virulence genes were found in our study (data not shown), the proportion of virulence genes associated with the *Firmicutes* increased from 31% to 60% of the virulence genes in the healthy and bleached libraries, respectively (data not shown). Therefore, temperature stress may indeed exacerbate disease-causing microbes that could, in turn, lead to secondary infections.

6.4.4 Shifts in the Eukaryotic microbial community

Sequences classified as *Fungi* increased in the bleached metagenome, which was consistent with results from Vega Thurber et al.'s (2009) experimental heat stress study. Although known to be a component of the healthy coral microbial community (Kendrick et al., 1982; Le Campion-Alsumard et al., 1995a,b; Bentis et al., 2000), the roles of *Fungi* in the coral holobiont remain unclear. *Fungi* can be pathogens of coral (Kim et al., 2000; 2006; Alker et al., 2001) and most *Fungi* affiliated sequences (98%) were assigned as *Ascomycota*, many of which are related to known plant pathogens, including *Marnaporthe grisea*, *Gibberella zeae* and *Eremothecium gossypii*. Other retrieved *Fungi* related sequences included *Schizosaccharomyces pombe*, *Neuropora crassa* and *Saccharomyces cerevisea*. Because many of these sequences were related to phytopathogenic *Fungi*, it is likely that the elevated abundance of *Fungi* within the bleached corals could have adversely affected their health during the period of bleaching.

Conversely, the proportion of sequences assigned to *Viridiplantae*, which includes the endolithic algae, *Ostreobium*, increased in the bleached metagenome. This is consistent with previous studies, which found that endolithic algae were highly active on bleached corals (Fine and Loya, 2002; Fine et al., 2004; Fine et al., 2005) and possibly serve as an alternative source of photosynthates during bleaching.

6.4.5 Low Archaeal sequence abundance

Very few *Archaeal* sequences were recovered from either the healthy or bleached metagenomes, suggesting that the *Archaea* may not be a dominant member of microbiota associated with the coral *A. millepora*. Of the archaeal sequences retrieved, *Euryarchaeota* and *Crenarchaeota*-like sequences were present and similar to those found in previous studies (Kellogg, 2004; Wegley et al., 2004; Vega Thurber et al.,

2009). *Crenarchaeota* sequences were higher in the bleached library, similarly to results of Vega Thurber et al.'s (2009) heat stress experiment, supporting the idea that elevated temperature enhances *Crenarchaeota* growth. As suggested by Vega Thurber et al. (2009), this may have implications for nitrogen cycling within the coral holobiont, given that these *Archaea* have ammonia oxidation genes (Beman et al., 2007). Furthermore, *Euryarchaeota* were reduced within the bleached library by 23.9%. *Euryarchaeota* include thermophiles, sulfur-metabolizing microorganisms and methanogens, and therefore reduction in these organisms may cause imbalances in biogeochemical cycles within the coral.

6.3.6 Elevated temperature increases coral associated viruses

As found by Wegley et al. (2007), phages were the most abundant viruses associated with *A. millepora*, and were predominately related to the *Microviridae* (Table 6.2). The proportion of *Microviridae* was significantly elevated in the bleached metagenome. It has been suggested that phages may aid in regulating the size of microbial populations on corals (Wegley et al., 2007), potentially contributing to the decrease in bacterial sequences recovered in the bleached metagenome. However, elevation in the proportion of *Microviridae* (15%) and the overall larger number of virus-like sequences in the bleached coral metagenome might also indicate that these viruses are not beneficial partners, with bleaching potentially leading to a diseased state. This possibility is supported by the studies of Vega-Thurber et al. (2008), who identified increases in the abundance of herpes-like viral sequences in *Porites compressa* coral colonies when stressed through exposure to reduced pH, elevated nutrients, and thermal stress. However, further specific studies on the role of viruses in the coral holobiont are required to confirm any cause/effect relationship between heat stress, viral populations and bleaching.

6.3.7 Data limitations

Despite extensive efforts to size fractionate samples and remove tissues associated with the coral host, the dominant proportion of sequences (55.3% and 86.4%) were assigned to the *Metazoa* for the healthy (i.e. 12995 sequences) and bleached (19017 sequences) coral metagenomes, respectively. Extensive improvements in fractionation methods are required to eliminate contaminating coral DNA so that more microbial reads can be generated. Special attention should be given to methods that remove mitochondria, which no doubt resulted in the bulk of DNA sequence reads, given similarities in their size range compared to targeted microbial cells. One of the strengths of this study was the ability to use coral fragments derived from an *in situ* bleaching event, rather than experimentally manipulated corals. These samples represented a unique resource because information on the history of their bleaching states, recovery, and photochemical efficiency was collected as part of a long term sampling and monitoring of the corals analysed (Bourne et al., 2008). However, the samples had been stored (at -80°C) for extended periods of time and both freezing and thawing likely contributed to the bursting of cells prior to fractionation, reducing target microbial reads and increasing contaminating coral DNA. Future work targeting fresh *in situ* bleached corals would be optimal, although predicting bleaching of individual coral colonies before an event is problematic and obtaining extensive environmental data is challenging. A recommended focus for future studies would be the use of metagenomics (or transcriptomics) to investigate the effects of bleaching on microbial communities associated with different coral microhabitats, such as coral tissue, mucus and skeleton. Separation of responses of microbial communities associated with each of these microhabitats is required before we can distinguish their individual contributions to coral health and thermal tolerance.

Additional limitations of our study included the possible inclusion of bias associated with whole genome amplification (Paez et al., 2004). Only small amounts of high quality total genomic DNA were retrieved after fractionation from each sample and therefore it was necessary to perform whole genomic amplification to provide enough DNA for pyrosequencing. Despite repeated attempts, this step was unavoidable. This method uses multiple displacement amplification (MPA), which can lead to random and uneven coverage of the whole genome resulting in variation in the abundance of sequences for each sample. To minimize the potential variability between the healthy and bleached metagenomes produced by MPA, we individually amplified 5 replicate samples before pooling and sequencing. We also chose to use GenomiPhi (Amersham Biosciences, Pittsburgh, PA), because it has been demonstrated to generate the least bias of the whole-genome amplification methods (Pinard et al., 2006).

A previous bacterial profiling study using a parallel set of coral samples from this collection clearly demonstrated shifts in the communities of bleached corals, which became dominated by *Vibrio*-affiliated sequences (Bourne et al., 2008). Similarly, other studies have shown an increase in *Vibrio* spp. for samples under heat stress or bleaching conditions (Ritchie et al., 2006; Chapter 2). In contrast, no significant shifts in *Vibrio* spp. affiliated sequences, either at the taxonomic or functional gene (virulence) classification levels, were detected in our study, but the low number of *Vibrio* affiliated sequences retrieved may have prevented detection of potential shifts. Alternatively, potential sequencing bias may have precluded detection of significant changes within this group, which could be addressed through further investigations and analysis of other large sequence datasets. Additional meta-transcriptomic approaches are required to identify whether this group contributes to

changes toward a more pathogenic bacterial community (as observed in Vega-Thurber et al., 2009) by determining if there is increased expression of virulence genes.

6.3.8 Conclusions

This study is the first to use a metagenomics approach to analyse changes in microbial associates of the coral holobiont following a natural, thermal bleaching event. We found that bleaching leads to increases in virulence genes and potentially pathogenic organisms, corroborating findings from controlled heat stress experiments (Vega-Thurber et al., 2009), although virulence genes were associated with *Firmicutes* rather than *Vibrio* species. Comparisons of healthy and bleached coral metagenomes also demonstrated alterations in sulfur, phosphate, carbohydrate and fatty acid cycling within the coral, verifying that biogeochemical cycling within the coral can be affected by environmental stress. We demonstrate that bleaching can cause shifts in all vital components of the coral microbial community, including *Bacteria*, micro-algae, *Fungi*, *Archaea* and viruses. Moreover, associated shifts in functional genes are likely to have had a detrimental effect on coral health during the bleaching event and on subsequent overall fitness following recovery. However, recovery of the corals suggests that the microbial shifts detected were not irreversible and the holobiont was resilient to the changes in associated microbial communities documented.

Chapter 7.0 General Discussion

7.1 Discussion

Studies presented in this thesis add to the growing body of evidence that bacteria are vital members of the coral holobiont. Given predictions of increasing degradation of coral reefs as the impacts of climate change accumulate, knowledge of the dynamics of bacterial associates will play an important role in understanding and predicting reef resilience to the onslaught of anthropogenic impacts, particularly those associated with rising seawater temperatures. The overarching aim of this thesis was to expand current knowledge of the dynamics of coral-associated bacteria under natural conditions and to explore how heat stress influences bacterial communities and coral holobiont fitness.

Results presented in Chapter 2 establish the first baseline for relationships between bacteria and different coral species on the Great Barrier Reef and identify factors contributing to the dynamics of these associations. Comparisons of the dominant ribotypes associated with three related species of *Acropora* at two different locations, demonstrate that the prominent bacteria are conserved between adjacent species, but differ between reef locations. Differences in the proximity of the two study reefs to an urban center suggest that terrestrial run-off may have contributed to the differences in bacterial constituents observed. These results challenge previous generalizations about the spatial stability of coral-bacteria relationships (Rohwer et al. 2002), but are supported by evidence of geographical differences in coral-associated bacteria in Curaçao (Klaus et al. 2005), Tobago (Guppy and Bythell 2006), and Taiwan (Hong et al. 2009). Moreover, comparisons among the microbial metagenomes of reefs in varying proximity to urban centers demonstrated that nearshore environments tend to harbour a higher abundance of bacteria, more heterotrophic bacteria and a greater number of potential pathogens than reefs distant to human population centres,

suggesting anthropogenic sources influence the microbial community of reef environments (Dinsdale et al. 2008). My study provides further support for the conclusion that environmental factors, such as land-based nutrient inputs, influence the structure of bacterial communities associated with corals.

Chapter 3 further illustrates how factors associated with the grow-out environment of corals can cause restructuring of the bacterial community. The presence of a metal ion source (Fe++) in the surrounding environment appears to have led to the establishment of dominant bacteria capable of metabolizing iron, bacteria which are not found under 'natural' conditions. The stability of these novel associations throughout the 1 year study also indicates that bacterial associations are flexible and can be sustained for many years with prolonged exposure to environmental factors. Further work should explore other environmental factors, such as nutrient inputs, pesticides and sediment that may contribute to changes in bacterial associates. Equally as important, future research should also investigate how these differences in community structure affect the functioning of the microbial community. For instance, it is possible that certain types of *Vibrio* sp. provide benefits to the coral by breaking down nutrients present in the seawater and passing these on to the coral animal. However, the dominance of other *Vibrio* sp. found on corals placed on metal racks indicates that corals are more susceptible to pathogenic infection during times of stress.

Another important issue that was presented in this study was characterization of the bacteria in early coral ontogeny. Establishing the onset of the association of the coral with the bacterial community is necessary for determining how this relationship is initiated and structured in the coral holobiont. Chapter 4 demonstrates that the microbial consortia associated with juvenile corals differed from that of adult corals at

nine months and further changed after one year. Furthermore, juvenile associates were far more diverse than adult communities and appeared to have little to no conserved structure at nine months. Bacterial communities became more structured at one year and patterns closer to those of adult corals emerged, indicating that young juvenile corals do not establish stable associations with bacteria. This suggests that a successional process takes place, whereby adult coral associates gradually replace the juvenile associated bacteria. The non-specific nature of bacterial associations at early developmental stages provides a mechanism by which a combination of bacterial associations can be formed that best suits the environmental conditions in which the coral resides. This may explain the differences in bacterial communities found in different reef environments (Chapter 2). However, further work needs to examine bacterial communities at different stages of coral development to determine how and when the adult community is established.

In Chapter 4 I also examined nine- month juvenile corals harbouring two different *Symbiodinium* types and could not discern any differences in the bacterial associated community harbouring type C1 or D *Symbiodinium*. However, when *Acropora tenuis* was sampled again three months later, there was proliferation of sequences closely related to *Vibrio corallyliiticus* in corals hosting type D, which was not observed in type C1 corals. This was attributed to the high temperatures experienced in the ambient environment during the time of collection. Corals hosting type D experienced high mortality rates and low growth rates, whereas those hosting type C1 did not, indicating suboptimal health states of corals when hosting *Symbiodinium* type D. Thus it appears that corals hosting *Symbiodinium* D were more susceptible to proliferation of an opportunistic pathogen during times of heat stress. This was further tested in Chapter 5 when one-year juveniles were placed in

experimental conditions of elevated temperature. Juvenile *A. tenuis* harbouring type C1 experienced minimal changes in bacterial associates when exposed to elevated temperature (32°C). Conversely, corals harbouring type D demonstrated dramatic increases in *Vibrio corallyliiticus* when subjected to higher temperatures but decrease of *Vibrio* sp. on corals placed at the control temperature (28°C). This suggests that corals were heat stressed in the field prior to collection, thus applying experimental heat stress increased the proliferation of the coral pathogen, *Vibrio corallyliiticus*. It is likely that this was opportunistic proliferation due to the compromised health of corals harbouring type D *Symbiodinium*. Taken together, these results provide evidence that the *Symbiodinium* type harboured is important for determining the resilience of the coral's bacterial community to heat stress. Thus, symbiont shuffling after stress events could mean the coral becomes more susceptible to bacterial infection.

In Chapter 6, a metagenomic approach was used to further investigate shifts in the taxonomic and functional components of the microbial community on corals during a natural bleaching event that occurred on the Great Barrier Reef in 2002. Comparison of healthy and bleached corals revealed that bleaching was correlated with shifts in all the vital components of the coral microbial community, including *Bacteria*, micro-algae, *Fungi*, *Archaea* and viruses. Significant increases in certain bacteria, fungi and viruses during heat stress indicate that microbial communities shift to more pathogenic members. In contrast to previous work, increases in *Vibrio* sp. were not detected on bleached corals; however there were significant increases in virulence genes. Increases were mostly attributed to *Firmicute* sequences, implicating this bacterium in pathogenesis. It is therefore possible that bleaching can lead to many types of potential infections. Examination of metabolic genes demonstrated that bleaching also altered the sulfur, phosphate, carbohydrate and fatty acid cycling within corals, verifying that

shifts in the microbial community can lead to alterations in biogeochemical cycling within corals during environmental stress. This may mean that as the coral is starved of photosynthates, the bacterial community turns to alternative sources of energy. Furthermore, secondary metabolism and stress genes increased on bleached corals, indicating that microbial constituents were experiencing stress. Therefore, the coral's bacterial community seems to have mechanisms for dealing with stress and certain microbial members may be resilient to high temperatures. These results demonstrate that metagenomics can be a useful approach for illustrating how bleaching is correlated with functional and structural shifts from a healthy mutualistic microbial community to a stressed pathogenic constituency.

7.2 Major Findings of this Thesis

1. The dominant coral-associated bacteria are the same on three closely-related, sympatic species of *Acropora* on the Great Barrier Reef, but bacterial associations differ between reef locations. Patterns in bacterial associations differed between reefs in the same manner for all three acroporid species (Ch. 2).

2. Bacterial communities associated with adult corals remain stable throughout the year at two reefs in the central section of the Great Barrier Reef (Ch. 2).

3. Environmental factors, such as elevated levels of iron, influence the type of bacteria harboured by corals. As an environmental driver of bacterial associations, elevated levels of iron may override the effects of mild temperature stress (Ch. 2 and Ch. 3).

4. Bacterial associates are not conserved on nine-month-old juvenile corals and differ from the bacterial associates of adult corals (Ch. 4).

5. There is no discernable relationship between type of *Symbiodinium* harboured and the structure of coral bacterial communities in nine-month-old juvenile corals (Ch. 4), which may largely reflect the lack of a stable bacterial association at this early stage of ontogeny (see point 4).

6. The *Symbiodinium* type hosted governs the susceptibility of corals to *Vibrio* sp. proliferation during heat stress (Ch. 4 and Ch. 5).

7. Coral bleaching is correlated with shifts in microbial structure and function. The metabolic capabilities of coral-associated microbes change from potentially beneficial to pathogenic (Ch. 6).

7.3 Future Work

Further studies of the roles and dynamics of coral microbial associates are crucial for enhancing current understanding of disease progression and the effects of climate change on coral reefs. While several studies, including this one, have provided knowledge of the diversity of coral-associated bacteria, we still have little understanding of the functioning of the corals' partners. Emerging technologies, such as metagenomics and metatranscriptomics, will be the next steps necessary to elucidate full community functioning. Chapter 6 illustrates how metagenomics can be used to explore changes in the functional capabilities, as well as taxonomic groups of microbial associates. Metatranscriptomics would further clarify which genes are being expressed, and therefore effectively show what the microbial constituents are doing on corals. Furthermore, it is important to link these developing sequencing and existing molecular techniques with single cell imaging, such as Flourescence *In Situ* Hybridization (FISH) and nano-sims, to visualize and map metabolic function at the single cell level. By applying these technologies to diseased and bleached corals, we

will be able to ascertain the roles of microbial consortia in the functioning of the coral holobiont and clarify the changes that occur in these relationships during disease progression and bleaching.

One issue from this study that remains unresolved concerns the role of *Vibrio* sp. in coral bleaching? In the metagenomics study, all bacteria affiliated with the the genus *Vibrio* were grouped together as species affiliation could not be discerned due to the short sequences generated. This can cause confusion as some strains of *Vibrio* sp. may be beneficial for the coral whereas others may be pathogenic. However, in the heat stress experiment the proliferation of *Vibrio coralliilyticus*, a known coral pathogen on bleached corals, implies that this species of bacteria is opportunistic and potentially pathogenic. It is possible that if the coral survives from bleaching that it may recover enough just to be subjected to future infection from bacterial pathogens. Longer-term studies would be necessary to determine whether recovered corals become victims of such opportunistic infection. What is more, previous studies have implicated *Vibrio* sp. as the cause of bleaching in certain instances (reviewed in Reshef et al. 2006). The work in this thesis has not proved or disproved this theory. Further work could investigate whether antibiotics could prevent bleaching in corals experiencing *Vibrio* sp. proliferation.

Two of the research questions addressed in this thesis remain unresolved, either because of inappropriate experimental protocols or temporal constraints in the timeframe of the study, and should be the subject of further research. Rather than determining if a change in environmental conditions between warmer and cooler reefs alters bacterial communities associated with corals (Chapter 3), it appears that the method used to attach corals to the reef during the translocation study (i.e. the use of metal racks) affected the bacterial communities present and confounded the results. To

determine how stable coral-associated bacterial communities are once adult associations are formed, future studies should investigate the effects of translocation to a new environment on coral-associated bacteria without using metal racks.

Although the study described in Chapter 4 revealed that juvenile corals host bacterial communities that are much more diverse than those described for adult corals, it was terminated before adult patterns of bacterial associations were detected. Thus this study did not fully describe ontogenetic changes in the diversity of bacterial associates. Further investigation of the onset adult patterns of microbial associations would refine understanding of possible competitive process that take place to establish the adult community, and potentially reveal which bacteria are necessary for coral development. Use of 16S rRNA pyrotag sequencing on samples throughout different stages of coral ontogeny would provide the depth of read necessary to capture the richness of bacterial diversity and the full development of microbial communities associated with corals.

Much of the work described in this thesis has demonstrated that the microbial community associated with coral is dynamic with different environmental conditions. Therefore, it would be prudent to examine other future scenarios that could potentially impact the coral holobiont. Ocean acidification for instance is likely to cause changes in the microbial community that may adversely affect coral health. Experiments simulating increased CO_2 could be used to examine the impacts of acidification on the coral's bacterial community. Similarly, the effects of pesticides and fertilizers from river run-off could lead to shifts in the coral's microbial community. Controlled experiments supplemented with samples collected from select environments could help us determine the impacts of run-off. A combination of different factors would also illustrate a closer approximation of what corals may be experiencing in the natural

environment; therefore complex experiments could be set up to simulate the compounded effects of increased temperature, pollution and ocean acidification.

This thesis has examined factors involved in structuring microbial communities associated with corals, the interactions that occur between bacteria and other microbial constituents, and how heat stress affects these associations and interactions. My results illustrate the complexity of microbial interactions that occur within the coral holobiont, and highlight that heat stress threatens the balance between microbial components in the coral holobiont. Future studies should focus on the functional aspects of these diverse microbial communities to determine what the dynamics of bacterial associations mean to the health of the holobiont in times of environmental stress.

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Appendix I. Affiliation of bacterial sequences retrieved from 16S rRNA gene clone libraries.

Closest relative*	Identity* %	Phylogenetic affiliation	M mil-1	M mil-2	M ten-1	M ten-2	M val-1	M val-2	O mil-1	O mil-2	O ten-1	O ten-2	O val-1
α-proteobacterium	99	*α-Proteobacteria*											
Bacterium DG941	98	*α-Proteobacteria*	14		9	5	4	24	1	1			
Bradyrhizobium sp.	99	*α-Proteobacteria*							2				
Brevundimonas sp.	100	*α-Proteobacteria*	1	2					3	6	5	17	12
Brevundimonas sp.	89	*α-Proteobacteria*								1			
Brevundimonas sp.	93	*α-Proteobacteria*								1			
Brevundimonas vesicularis	95	*α-Proteobacteria*								1			
Caulobacter	97	*α-Proteobacteria*											1
Caulobacteraceae bact	99	*α-Proteobacteria*				1					1		
Caulobacterales	99	*α-Proteobacteria*					2						
Leisingera aquamarina	99	*α-Proteobacteria*											
Loktanella koreensis	95	*α-Proteobacteria*						1					
Novosphingobium aromaticivorans	98	*α-Proteobacteria*										2	
Oceanicola batsensis	96	*α-Proteobacteria*						1					
Paracoccus kamogawaensis	76	*α-Proteobacteria*				1							
Pseudoruegeria aquimaris	98	*α-Proteobacteria*		1			1						
Rhodobacter	92	*α-Proteobacteria*						1					
Rhodobacteraceae	90	*α-Proteobacteria*						1					
Rhodobacteraceae bacterium	97	*α-Proteobacteria*							1	1			
Rhodobacteraceae bacterium	95	*α-Proteobacteria*											1
Roseobacter sp.	93	*α-Proteobacteria*				1							
Roseobacter sp.	100	*α-Proteobacteria*	13	21	7	1	11	4	4	1	57		
Ruegeria sp.	98	*α-Proteobacteria*										4	
Silicibacter lacuscaerulensis	99	*α-Proteobacteria*								1		2	
Sphingobium sp.	99	*α-Proteobacteria*											
Sphingomonas sp.	99	*α-Proteobacteria*											1
Thalassobius mediterraneus	99	*α-Proteobacteria*		1					1			1	
Thalassococcus halodurans	99	*α-Proteobacteria*		1									
Unc *α*-proteobacterium	94	*α-Proteobacteria*					2						

Unc *α*-proteobacterium	91	*α-Proteobacteria*				1								
Unc *α*-proteobacterium	95	*α-Proteobacteria*				6								
Unc *α*-proteobacterium	97	*α-Proteobacteria*						1						
Unc *α*-proteobacterium	90	*α-Proteobacteria*		1										
Unc *α*-proteobacterium	99	*α-Proteobacteria*		2										
Unc *α*-proteobacterium	97	*α-Proteobacteria*		1										
Unc *α*-proteobacterium	90	*α-Proteobacteria*								1				
Unc *α*-proteobacterium	92	*α-Proteobacteria*											1	
Unc *α*--proteobacterium	95	*α-Proteobacteria*	4	1				4						
Unc bacterium	98	*α-Proteobacteria*												
Unc bacterium	98	*α-Proteobacteria*												
Unc *Caulobacterales*	99	*α-Proteobacteria*							11					
Bacteroidetes	95	*Bacteroidetes*												
Microscilla aggregans	92	*Bacteroidetes*									1			
Unc *Flavobacteria*	95	*Bacteroidetes*							1					
Achromobacter xylosoxidans	99	*β-Proteobacteria*			1			1	4	4	3		1	
Alcaligenes sp.	98	*β-Proteobacteria*						1						
Comamonas acidovorans	99	*β-Proteobacteria*												
Delftia sp.	99	*β-Proteobacteria*	1						1				3	
Delftia sp.	93	*β-Proteobacteria*												
Diaphorobacter oryzae	99	*β-Proteobacteria*											1	
Unc bacterium	99	*β-Proteobacteria*							5					
Unc *β*-proteobacterium	99	*β-Proteobacteria*								11				
Spirulina subsalsa	98	*Cyanobacteria*			11									
Anaeromyxobacter dehalogenans	97	*δ-Proteobacteria*	2	2		2								
Anaeromyxobacter sp.	94	*δ-Proteobacteria*	3	2										
Anaeromyxobacter sp.	90	*δ-Proteobacteria*						1						
δ - proteobacterium	98	*δ-Proteobacteria*		3										
δ - proteobacterium	98	*δ-Proteobacteria*	1											
δ - proteobacterium	89	*δ-Proteobacteria*		4										
δ - proteobacterium	91	*δ-Proteobacteria*	1											
Myxobacterium	98	*δ-Proteobacteria*	2				1							
Unc δ- proteobacterium	96	*δ-Proteobacteria*				1								

	Bacterium PS7	93	*Firmicutes*									1		2
	Marine bacterium	94	*Firmicutes*						1					
	Acinetobacter sp.	99	*γ-Proteobacteria*				4	6	3					1
	Alteromonas sp.	99	*γ-Proteobacteria*							1				1
	Bacterium c1cc54	96	*γ-Proteobacteria*			9								
	Enterobacter aerogenes	99	*γ-Proteobacteria*											
	Escherichia coli	100	*γ-Proteobacteria*											
	Ferrimonas kyonanensis	98	*γ-Proteobacteria*											
	γ-proteobacterium	98	*γ-Proteobacteria*				2							
	γ-proteobacterium	90	*γ-Proteobacteria*						1					
	γ-proteobacterium	94	*γ-Proteobacteria*						3					
	γ-proteobacterium	99	*γ-Proteobacteria*					1	1	15		3	12	
	Idiomarina sp.	98	*γ-Proteobacteria*											5
	Marinobacter sp.	77	*γ-Proteobacteria*				1							
	Marinobacter sp.	99	*γ-Proteobacteria*	13	12	13	11	10	6	1				
	Pseudoalteromonas sp.	100	*γ-Proteobacteria*											3
	Pseudomonas sp.	99	*γ-Proteobacteria*								2		4	
	Serratia marcescens	99	*γ-Proteobacteria*							2	2			1
	Shigella sp.	99	*γ-Proteobacteria*											
	Spongiobacter nickelotolerans	97	*γ-Proteobacteria*	12	16	9	21	14	14	1				1
	Spongiobacter sp.	94	*γ-Proteobacteria*			7	2	12		1				
	Spongiobacter sp.	91	*γ-Proteobacteria*		3									
	Spongiobacter sp.	93	*γ-Proteobacteria*			2								
	Stenotrophomonas maltophilia	99	*γ-Proteobacteria*	6	6	3	6	4	2	8	20	7	14	3
	Stenotrophomonas sp.	93	*γ-Proteobacteria*	1	1		1				1			
	Stenotrophomonas sp.	90	*γ-Proteobacteria*		1									
	Unc α-proteobacterium	95	*γ-Proteobacteria*				3							
	Unc bacterium	92	*γ-Proteobacteria*			1								
	Unc bacterium	96	*γ-Proteobacteria*		1									
	Unc bacterium	92	*γ-Proteobacteria*		1									
	Unc bacterium	96	*γ-Proteobacteria*		1									
	Unc bacterium	97	*γ-Proteobacteria*		2									
	Unc bacterium	98	*γ-Proteobacteria*		3									

	Unc bacterium	96	*γ-Proteobacteria*								1				
	Unc γ- proteobacterium	94	*γ-Proteobacteria*				3								
	Unc γ- proteobacterium	94	*γ-Proteobacteria*					1							
	Unc γ- proteobacterium	95	*γ-Proteobacteria*			4									
	Unc γ- proteobacterium	99	*γ-Proteobacteria*												
	Vibrio coralliilyticus	99	*γ-Proteobacteria*				1								
	Vibrio coralliilyticus	93	*γ-Proteobacteria*				1								
	Vibrio corallilyticus	98	*γ-Proteobacteria*	1	2			4							
	Vibrio harveyi	99	*γ-Proteobacteria*											1	
	Vibrio sp.	99	*γ-Proteobacteria*	5	3	3	6	3	2			7	3		
	Bacterium 2-4	99	Unclassified								1				
	Bacterium s1cb31	99	Unclassified						6		3				
	Marine bacterium	99	Unclassified									1			
	Mucus bacterium	94	Unclassified	3			1								
	Unc bacterium	92	Unclassified												
	Unc bacterium	99	Unclassified												
	Unc bacterium	93	Unclassified												
	Unc bacterium	90	Unclassified												
	Unc bacterium	99	Unclassified										1		
	Unc bacterium	99	Unclassified			1									
	Unc bacterium	83	Unclassified												
	Unc bacterium	98	Unclassified												
	Unc bacterium	92	Unclassified						1						
	Unc bacterium	94	Unclassified												
	Unc bacterium	99	Unclassified	1											
	Unc bacterium	91	Unclassified								1				
	Unc bacterium	95	Unclassified				1	4	3						
	Unc bacterium	99	Unclassified				1								
	Unc bacterium	98	Unclassified	6					1						
	Unc bacterium	92	Unclassified						3						
	Unc bacterium	95	Unclassified					2							
	Unc bacterium	95	Unclassified						1						
	Unc bacterium	98	Unclassified												

	Unc bacterium	99	Unclassified										1		
	Unc bacterium	99	Unclassified								1				
	Unc bacterium	99	Unclassified										5		
	Unc bacterium	99	Unclassified							1					
	Unc bacterium	97	Unclassified			3									
	Unc bacterium	95	Unclassified												
	Unc bacterium	98	Unclassified					2	1						
	Unc bacterium	100	Unclassified												
	Unc bacterium	98	Unclassified												
	Unc bacterium	99	Unclassified							3	4				
	Unc bacterium	99	Unclassified								4				
	Unc bacterium	96	Unclassified												
	Unc bacterium	99	Unclassified											1	
	Unc bacterium	99	Unclassified									4	12		
	Unc bacterium	88	Unclassified								1				
	Unc bacterium	100	Unclassified											2	
	Unc bacterium	95	Unclassified			1	2								
	Unc bacterium	99	Unclassified												
	Unc bacterium	99	Unclassified											1	
	Unc bacterium	99	Unclassified											2	
	Unc bacterium	99	Unclassified												
	Unc bacterium	99	Unclassified												
	Unc bacterium	99	Unclassified								1				
	Unc bacterium	98	Unclassified								1				
	Unc bacterium	92	Unclassified						1						
	Unc bacterium	99	Unclassified										1		
	Unc bacterium	96	Unclassified				1								
	Unc bacterium	98	Unclassified								1				
	Unc bacterium	99	Unclassified							7					
	Unc bacterium	92	Unclassified									1			
	Unc bacterium	92	Unclassified				1								
	Unc bacterium	98	Unclassified								1				
	Unc bacterium	95	Unclassified				1								

	Unc bacterium	98	Unclassified			1								
	Unc bacterium	92	Unclassified											
	Unc bacterium	99	Unclassified			1								
	Unc bacterium	99	Unclassified											
	Unc bacterium	99	Unclassified								7			
	Unc bacterium	98	Unclassified											1
	Unc bacterium	99	Unclassified										1	
	Unc bacterium	98	Unclassified								1			
	Unc bacterium	100	Unclassified				1							
	Unc bacterium	99	Unclassified			1		1						
	Unc bacterium	97	Unclassified			4			2					
	Unc bacterium	99	Unclassified											
	Unc bacterium	95	Unclassified	3			2							5
	Unc bacterium	98	Unclassified							1				
	Unc bacterium	97	Unclassified				1							2
	Total Clones			**93**	**94**	**91**	**93**	**85**	**92**	**75**	**82**	**91**	**80**	**53**

* Sequences were aligned to the closest relative over 700bp using BLAST (Zhang et al., 2000). The similarity was calculated with gaps not taken into account.

M mil, Magnetic Island *A. millepora*;

M ten, Magnetic Island *A. tenuis*;

M val, Magnetic Island *A. valida*;

O mil, Orpheus Island *A. millepora*;

O ten, Orpheus Island *A. tenuis*;

O val, Orpheus Island *A. valida*.

S mil, *A. millepora* collected in summer 2007,

W mil, *A. millepora* collected in winter 2008

Appendix II. Affiliation of bacterial sequences retrieved from juvenile coral 16S rRNA gene clone libraries.

Acc. #	clone	Closest Relative*	Ident.*	Affiliation	MC1	MC2	MD1	MD2	TC1	TC2	TD1	TD2	STC1	STC2	STD1	STD2
GQ301209	OTU-001	*Acidobacteria* (DQ289940)	98%	*Acidobacteria*						2	1					
GQ301210	OTU-002	*Actinobacterium* (AY225656)	92%	*Actinobacteria*						1						
GQ301330	OTU-003	*Bacteroidetes* (EF016472)	91%	*Bacteroidetes*										1	2	2
GQ301331	OTU-004	*Bacteroidetes* (AY162097)	99%	*Bacteroidetes*			1	1	2	3						
GQ301332	OTU-005	*Cellulophaga pacifica* (AB100842)	91%	*Bacteroidetes*	1											
GQ301333	OTU-006	*Cytophaga* (AB073566)	97%	*Bacteroidetes*											2	
GQ301334	OTU-007	*Flavobacteria* (AF277542)	98%	*Bacteroidetes*	4		1	2	1	1	1		4	2		
GQ301335	OTU-008	*Flexibacter* (AB058905)	99%	*Bacteroidetes*											2	
GQ301336	OTU-009	*Muricauda* sp. (AY576744)	99%	*Bacteroidetes*	2		1		2				1	1		
GQ301337	OTU-010	*Sphingobacteria* (AY317117)	93%	*Bacteroidetes*	1				1							
GQ301476	OTU-011	Unc bacterium (EF378470)	97%	*Bacteroidetes*^	1								3			
GQ301343	OTU-012	*cyanobacterium* (AY580402)	98%	*Cyanobacteria*				2								
GQ301344	OTU-013	*cyanobacterium* (AY702182)	97%	*Cyanobacteria*								1		1		
GQ301345	OTU-014	*Leptolyngbya* sp. (AF132786)	92%	*Cyanobacteria*								1				
GQ301346	OTU-015	*Lyngbya hieronymusii* (AB045906)	93%	*Cyanobacteria*	3											
GQ301347	OTU-016	*Oscillatoria* sp. (AB058224)	96%	*Cyanobacteria*	1		2	2								
GQ301348	OTU-017	*Pleurocapsa* sp. (X78681)	99%	*Cyanobacteria*	1											
GQ301349	OTU-018	*Pseudanabaena* (AB039018)	98%	*Cyanobacteria*	1											
GQ301350	OTU-019	*Spirulina* sp. (AF091109)	98%	*Cyanobacteria*		1										
GQ301351	OTU-020	*Synechococcus* sp. (AF132772)	91%	*Cyanobacteria*	4			1								
GQ301440	OTU-021	Unc bacterium (AJ538357)	99%	*Cyanobacteria*^						1						
GQ301367	OTU-022	*Caldicellulosiruptor lactoaceticus* (X82842)	99%	*Firmicutes*			1									
GQ301368	OTU-023	Gram-positive bacterium (AF424415)	95%	*Firmicutes*	1		2									
GQ301411	OTU-024	Bacterium DG1026 (AY258098)	97%	Unclassified	1		2									
GQ301412	OTU-025	Bacterium DG941 (AY258087)	98%	Unclassified						1						
GQ301413	OTU-026	Bacterium DG981 (AY258094)	98%	Unclassified	1					1						
GQ301415	OTU-028	Bacterium S1cc11 (DQ416573)	99%	Unclassified							1					
GQ301416	OTU-029	Bacterium S1cc93 (DQ416602)	96%	Unclassified						1						
GQ301417	OTU-030	Bacterium S1cc96 (DQ416604)	95%	Unclassified						1						
GQ301418	OTU-031	Bacterium SE4 (EU520344)	99%	Unclassified					1							
GQ301419	OTU-032	Bacterium SM17-19 (AY773149)	96%	Unclassified					1							
GQ301420	OTU-033	eubacterium (U87508)	94%	Unclassified			1									
GQ301421	OTU-034	marine eubacterium (AF159654)	97%	Unclassified	1											
GQ301423	OTU-035	Mucus bacterium (AY654804)	98%	Unclassified		1										
GQ301426	OTU-036	Mucus bacterium (AY654836)	93%	Unclassified		1										
GQ301425	OTU-037	Mucus bacterium (AY654813)	94%	Unclassified				1								
GQ301496	OTU-038	Unc bacterium (AY133399)	92%	Unclassified	1											

GQ301499	OTU-039	Unc bacterium (AY568766)	95%	Unclassified	1											
GQ301523	OTU-040	Unc bacterium (AY493913)	98%	Unclassified	1											
GQ301502	OTU-041	Unc bacterium (AY942756)	95%	Unclassified	1											
GQ301495	OTU-043	Unc bacterium (AF382114)	95%	Unclassified	1											
GQ301488	OTU-044	Unc bacterium (AB234238)	92%	Unclassified	1											
GQ301494	OTU-045	Unc bacterium (AF332287)	94%	Unclassified	1											
GQ301517	OTU-047	Unc bacterium (AB234238)	92%	Unclassified	1											
GQ301505	OTU-048	Unc bacterium (DQ017948)	91%	Unclassified	1											
GQ301509	OTU-049	Unc bacterium (DQ816499)	89%	Unclassified		1										
GQ301513	OTU-050	Unc bacterium (EF516738)	97%	Unclassified		1										
GQ301441	OTU-051	Unc bacterium (AJ617857)	98%	Unclassified		2							1			
GQ301520	OTU-052	Unc bacterium (DQ330995)	95%	Unclassified		2										
GQ301512	OTU-053	Unc bacterium (EF378470)	97%	Unclassified		1										
GQ301507	OTU-054	Unc bacterium (DQ345638)	91%	Unclassified			1									
GQ301453	OTU-056	Unc bacterium (AM176885)	95%	Unclassified			1		1							
GQ301489	OTU-057	Unc bacterium (AB266994)	94%	Unclassified			1									
GQ301503	OTU-058	Unc bacterium (AY942776)	97%	Unclassified			2	5								
GQ301492	OTU-059	Unc bacterium (AJ966605)	93%	Unclassified				1								
GQ301504	OTU-060	Unc bacterium (DQ004765)	93%	Unclassified				1								
GQ301491	OTU-061	Unc bacterium (AJ966596)	92%	Unclassified			1	2								
GQ301518	OTU-062	Unc bacterium (DQ330954)	97%	Unclassified				1								
GQ301435	OTU-063	Unc bacterium (AY913368)	93%	Unclassified				1								
GQ301500	OTU-064	Unc bacterium (AY568808)	95%	Unclassified				4								
GQ301490	OTU-065	Unc bacterium (AJ863221)	94%	Unclassified				1								
GQ301508	OTU-066	Unc bacterium (DQ791471)	99%	Unclassified				1								
GQ301433	OTU-067	Unc bacterium (EF613736)	97%	Unclassified				1								
GQ301516	OTU-068	Unc bacterium (EU290675)	97%	Unclassified				1								
GQ301449	OTU-069	Unc bacterium (AY845230)	90%	Unclassified					1							
GQ301478	OTU-070	Unc bacterium (EF613736)	99%	Unclassified					2		1					
GQ301479	OTU-071	Unc bacterium (EF658677)	99%	Unclassified					1							
GQ301475	OTU-072	Unc bacterium (EF208711)	99%	Unclassified					2							
GQ301439	OTU-073	Unc bacterium (AB329999)	97%	Unclassified					1							
GQ301480	OTU-074	Unc bacterium (EU050885)	96%	Unclassified					1							
GQ301524	OTU-075	Unc bacterium (Z88572)	98%	Unclassified					1							
GQ301498	OTU-076	Unc bacterium (AY529883)	99%	Unclassified					1	1						
GQ301521	OTU-077	Unc bacterium (EF702033)	95%	Unclassified					1							
GQ301514	OTU-078	Unc bacterium (EF574958)	92%	Unclassified					1							
GQ301456	OTU-079	Unc bacterium (DQ256660)	98%	Unclassified					1							
GQ301462	OTU-080	Unc bacterium (DQ416532)	96%	Unclassified					1							
GQ301477	OTU-081	Unc bacterium (EF572884)	98%	Unclassified					1							
GQ301447	OTU-082	Unc bacterium (AY529883)	99%	Unclassified						2						
GQ301446	OTU-083	Unc bacterium (AY373415)	93%	Unclassified						1						
GQ301432	OTU-084	Unc bacterium (DQ395405)	93%	Unclassified						1						
GQ301527	OTU-085	Unc bacterium (AF365733)	96%	Unclassified						1						
GQ301452	OTU-086	Unc bacterium (DQ154842)	94%	Unclassified						1						

GQ301429	OTU-088	Unc bacterium (AY654762)	99%	Unclassified						1						
GQ301428	OTU-089	Unc bacterium (AY654756)	94%	Unclassified						1						
GQ301430	OTU-090	Unc bacterium (AY654804)	90%	Unclassified						1						
GQ301468	OTU-091	Unc bacterium (DQ836762)	97%	Unclassified						1						
GQ301457	OTU-092	Unc bacterium (DQ300623)	96%	Unclassified						2						
GQ301434	OTU-093	Unc bacterium (U78037)	98%	Unclassified						1						
GQ301487	OTU-094	Unc bacterium (AB100008)	96%	Unclassified						1						
GQ301501	OTU-095	Unc bacterium (AY654813)	94%	Unclassified						1						
GQ301485	OTU-097	Unc bacterium (EU491423)	96%	Unclassified									1			
GQ301467	OTU-099	Unc bacterium (DQ819004)	95%	Unclassified									1			
GQ301481	OTU-100	Unc bacterium (EU183892)	94%	Unclassified									1			
GQ301455	OTU-103	Unc bacterium (DQ256654)	99%	Unclassified										1		
GQ301484	OTU-104	Unc bacterium (EU491359)	96%	Unclassified										1		
GQ301442	OTU-105	Unc bacterium (AF365559)	96%	Unclassified										1		
GQ301482	OTU-107	Unc bacterium (EU287124)	93%	Unclassified								1				
GQ301464	OTU-109	Unc bacterium (DQ451520)	97%	Unclassified								1				
GQ301438	OTU-110	Unc bacterium (AB274847)	95%	Unclassified								2				
GQ301466	OTU-111	Unc bacterium (DQ814239)	92%	Unclassified								1				
GQ301458	OTU-113	Unc bacterium (DQ300780)	90%	Unclassified								1				
GQ301515	OTU-116	Unc bacterium (EF613736)	95%	Unclassified							1					
GQ301511	OTU-117	Unc bacterium (EF125410)	90%	Unclassified							1					
GQ301463	OTU-119	Unc bacterium (DQ438280)	96%	Unclassified							1	2				
GQ301445	OTU-120	Unc bacterium (AY328849)	99%	Unclassified							1					
GQ301437	OTU-123	Unc bacterium (AB126359)	98%	Unclassified												1
GQ301483	OTU-124	Unc bacterium (EU438568)	98%	Unclassified												1
GQ301448	OTU-125	Unc bacterium (AY654772)	94%	Unclassified												1
GQ301471	OTU-127	Unc bacterium (EF040559)	95%	Unclassified											1	
GQ301454	OTU-128	Unc bacterium (DQ234649)	91%	Unclassified											1	
GQ301443	OTU-130	Unc bacterium (AF468246)	94%	Unclassified											1	
GQ301460	OTU-131	Unc bacterium (DQ395000)	98%	Unclassified											1	
GQ301451	OTU-132	Unc bacterium (AY942776)	97%	Unclassified						1					2	
GQ301450	OTU-134	Unc bacterium (AY942756)	92%	Unclassified											1	
GQ301465	OTU-135	Unc bacterium (DQ513853)	95%	Unclassified											1	
GQ301474	OTU-136	Unc bacterium (EF173618)	93%	Unclassified											1	
GQ301459	OTU-138	Unc bacterium (DQ300840)	91%	Unclassified											1	
GQ301444	OTU-139	Unc bacterium (AY193225)	97%	Unclassified											1	
GQ301472	OTU-140	Unc bacterium (EF089463)	93%	Unclassified											1	
GQ301473	OTU-141	Unc bacterium (EF125443)	90%	Unclassified											1	
GQ301469	OTU-142	Unc bacterium (DQ985940)	92%	Unclassified											1	
GQ301211	OTU-144	*Agrobacterium* sp. (AB247617)	99%	*α-proteobacteria*	2			1			1					
GQ301212	OTU-145	*Ahrensia* sp. (AJ582086)	97%	*α-proteobacteria*			1	1								
GQ301303	OTU-146	*Azospirillum amazonense* (Z29616)	89%	*α-proteobacteria*				1								
GQ301304	OTU-148	*Brevundimonas nasdae* (AB426561)	99%	*α-proteobacteria*				1	11	1	22	19	5	1	1	1
GQ301305	OTU-149	*Caulobacteraceae* (DQ857204)	99%	*α-proteobacteria*	2	1				4		7				

GQ301306	OTU-150	*Erythrobacter* sp. (DQ985055)	98%	α-*proteobacteria*		2	2									1
GQ301307	OTU-151	*Hyphomicrobiaceae* (EF019026)	93%	α-*proteobacteria*												1
GQ301308	OTU-152	*Hyphomonas oceanitis* (AF082797)	96%	α-*proteobacteria*				1								
GQ301309	OTU-153	*Kordiimonas* sp. (AY682384)	93%	α-*proteobacteria*	1					2						
GQ301310	OTU-154	*Leisingera aquamarina* (AM900415)	99%	α-*proteobacteria*					1							
GQ301311	OTU-155	*Mesorhizobium* sp. (DQ917826)	98%	α-*proteobacteria*				3								2
GQ301312	OTU-157	*Paracoccus* sp. (EU375176)	95%	α-*proteobacteria*												1
GQ301313	OTU-158	*Phenylobacterium* sp. (AY534887)	98%	α-*proteobacteria*								2				
GQ301526	OTU-160	*Rhizobium* sp. (AB069651)	100%	α-*proteobacteria*									1		1	
GQ301315	OTU-161	*Rhodobacteraceae* (AY962292)	99%	α-*proteobacteria*	4					1						
GQ301316	OTU-162	*Rhodobacteraceae* (DQ486507)	98%	α-*proteobacteria*										1		
GQ301314	OTU-163	*Rhodobacteraceae* (AM990709)	100%	α-*proteobacteria*										1		
GQ301317	OTU-164	*Rhodobacteraceae* (EF629850)	99%	α-*proteobacteria*										1		
GQ301318	OTU-165	*Rhodopseudomonas julia* (AY428572)	93%	α-*proteobacteria*				4								
GQ301319	OTU-166	*Rhodovulum* sp. (DQ868668)	97%	α-*proteobacteria*					1							
GQ301320	OTU-167	*Roseibium hamelinense* (D85836)	95%	α-*proteobacteria*				1								
GQ301321	OTU-168	*Roseobacter* sp. (AY745856)	99%	α-*proteobacteria*					1	1	3	3		2	3	
GQ301322	OTU-169	*Roseovarius aestuarii* (EU156066)	97%	α-*proteobacteria*				1						1		
GQ301323	OTU-170	*Ruegeria* sp. (AJ391197)	99%	α-*proteobacteria*				3		1					5	
GQ301324	OTU-171	*Silicibacter* sp. (AY369990)	99%	α-*proteobacteria*	1			3							1	
GQ301325	OTU-172	*Sinorhizobium* sp. (AJ879127)	98%	α-*proteobacteria*				1								
GQ301326	OTU-173	*Sphingomonas sp.* (EU817494)	99%	α-*proteobacteria*									4	1		1
GQ301327	OTU-174	*Stappia alba* (AJ889010)	99%	α-*proteobacteria*	1		1									
GQ301328	OTU-175	Unc bacterium (AJ009467)	94%	α-*proteobacteria*	1											
GQ301329	OTU-176	Unc bacterium (AY700606)	100%	α-*proteobacteria*			1									
GQ301282	OTU-177	Unc α-*proteobacterium* (AY499896)	95%	α-*proteobacteria*	6			1								
GQ301283	OTU-178	Unc α-*proteobacterium* (AY499897)	98%	α-*proteobacteria*						1						
GQ301293	OTU-179	Unc α-*proteobacterium* (DQ289935)	94%	α-*proteobacteria*	1											
GQ301251	OTU-180	Unc α-*proteobacterium* (DQ889882)	96%	α-*proteobacteria*	1											
GQ301297	OTU-181	Unc α-*proteobacterium* (DQ888854)	100%	α-*proteobacteria*	2											
GQ301285	OTU-182	Unc α-*proteobacterium* (AY580541)	94%	α-*proteobacteria*	1											
GQ301274	OTU-184	Unc α-*proteobacterium* (AJ890099)	99%	α-*proteobacteria*	3		1									
GQ301299	OTU-185	Unc α-*proteobacterium* (EF123328)	94%	α-*proteobacteria*		2										
GQ301296	OTU-186	Unc α-*proteobacterium* (DQ811849)	97%	α-*proteobacteria*		1										
GQ301291	OTU-187	Unc α-*proteobacterium* (DQ269055)	96%	α-*proteobacteria*		1										
GQ301295	OTU-188	Unc α-*proteobacterium* (DQ446161)	99%	α-*proteobacteria*		1										
GQ301292	OTU-189	Unc α-*proteobacterium* (DQ289904)	93%	α-*proteobacteria*		1	2									
GQ301279	OTU-190	Unc α-*proteobacterium* (AF253467)	97%	α-*proteobacteria*			1									

GQ301267	OTU-191	Unc α-*proteobacterium* (AJ633940)	99%	α-*proteobacteria*			6									
GQ301286	OTU-193	Unc α-*proteobacterium* (AY711964)	94%	α-*proteobacteria*			2									
GQ301290	OTU-194	Unc α-*proteobacterium* (DQ200607)	98%	α-*proteobacteria*			1									
GQ301278	OTU-195	Unc α-*proteobacterium* (AF190209)	99%	α-*proteobacteria*			1									
GQ301280	OTU-197	Unc α-*proteobacterium* (AF406524)	93%	α-*proteobacteria*				1								
GQ301288	OTU-198	Unc α-*proteobacterium* (DQ070827)	92%	α-*proteobacteria*				2								
GQ301302	OTU-199	Unc α-*proteobacterium* (AY162081)	94%	α-*proteobacteria*				1								
GQ301268	OTU-200	Unc α-*proteobacterium* (AJ633952)	92%	α-*proteobacteria*				1								
GQ301270	OTU-201	Unc α-*proteobacterium* (AJ633970)	94%	α-*proteobacteria*				1								
GQ301275	OTU-202	Unc α-*proteobacterium* (AM162572)	89%	α-*proteobacteria*				3								
GQ301222	OTU-203	Unc α-*proteobacterium* (AJ633984)	98%	α-*proteobacteria*				1								
GQ301273	OTU-204	Unc α-*proteobacterium* (AJ890097)	98%	α-*proteobacteria*				1								
GQ301266	OTU-205	Unc α-*proteobacterium* (AB015579)	88%	α-*proteobacteria*				1								
GQ301289	OTU-206	Unc α-*proteobacterium* (DQ070828)	94%	α-*proteobacteria*			1	1								
GQ301269	OTU-207	Unc α-*proteobacterium* (AJ633963)	94%	α-*proteobacteria*				1								
GQ301284	OTU-208	Unc α-*proteobacterium* (AY580443)	97%	α-*proteobacteria*				1								
GQ301277	OTU-209	Unc α-*proteobacterium* (AM709743)	91%	α-*proteobacteria*				1								
GQ301228	OTU-210	Unc α-*proteobacterium* (AF384141)	96%	α-*proteobacteria*				1								
GQ325717	OTU-211	Unc α-*proteobacterium* (DQ860071)	98%	α-*proteobacteria*				1								
GQ301298	OTU-212	Unc α-*proteobacterium* (DQ889913)	94%	α-*proteobacteria*				1								
GQ301276	OTU-213	Unc α-*proteobacterium* (AM259856)	96%	α-*proteobacteria*				1								
GQ301249	OTU-214	Unc α-*proteobacterium* (DQ431900)	99%	α-*proteobacteria*					1							
GQ301256	OTU-215	Unc α-*proteobacterium* (EF123411)	99%	α-*proteobacteria*						1						
GQ301247	OTU-216	Unc α-*proteobacterium* (DQ289904)	98%	α-*proteobacteria*					1					1		
GQ301246	OTU-217	Unc α-*proteobacterium* (DQ289901)	98%	α-*proteobacteria*					1							
GQ301242	OTU-218	Unc α-*proteobacterium* (DQ107390)	99%	α-*proteobacteria*					1	2						
GQ301248	OTU-219	Unc α-*proteobacterium* (DQ289935)	97%	α-*proteobacteria*					1	1						
GQ301226	OTU-221	Unc α-*proteobacterium* (AM930446)	100%	α-*proteobacteria*					2							
GQ301294	OTU-222	Unc α-*proteobacterium* (DQ446103)	98%	α-*proteobacteria*					1							
GQ301217	OTU-223	Unc α-*proteobacterium* (AJ633952)	97%	α-*proteobacteria*						1						
GQ301237	OTU-224	Unc α-*proteobacterium* (AY795760)	94%	α-*proteobacteria*						1						
GQ301272	OTU-225	Unc α-*proteobacterium* (AJ890096)	92%	α-*proteobacteria*						1						

GQ301243	OTU-226	Unc α-*proteobacterium* (DQ200417)	98%	α-*proteobacteria*						1						
GQ301287	OTU-227	Unc α-*proteobacterium* (AY922212)	96%	α-*proteobacteria*						2						
GQ301235	OTU-228	Unc α-*proteobacterium* (AY711161)	99%	α-*proteobacteria*						1						
GQ301244	OTU-229	Unc α-*proteobacterium* (DQ200634)	96%	α-*proteobacteria*						1						
GQ301281	OTU-230	Unc α-*proteobacterium* (AF424272)	97%	α-*proteobacteria*						1	1					
GQ301250	OTU-231	Unc α-*proteobacterium* (DQ860065)	96%	α-*proteobacteria*						1						
GQ301239	OTU-232	Unc α-*proteobacterium* (AY922212)	96%	α-*proteobacteria*						1						
GQ301218	OTU-233	Unc α-*proteobacterium* (AJ633953)	98%	α-*proteobacteria*						1						
GQ301238	OTU-234	Unc α-*proteobacterium* (AY921824)	99%	α-*proteobacteria*						1						
GQ301233	OTU-235	Unc α-*proteobacterium*	98%	α-*proteobacteria*						1						
GQ301229	OTU-237	Unc α-*proteobacterium* (AF424276)	95%	α-*proteobacteria*						1						
GQ301236	OTU-238	Unc α-*proteobacterium* (AY711774)	94%	α-*proteobacteria*						1						
GQ301214	OTU-239	Unc α-*proteobacterium* (AB116473)	98%	α-*proteobacteria*						1						
GQ301213	OTU-240	Unc α-*proteobacterium* (AB015526)	91%	α-*proteobacteria*						1						
GQ301215	OTU-241	Unc α-*proteobacterium* (AB247855)	97%	α-*proteobacteria*						1						
GQ301271	OTU-242	Unc α-*proteobacterium* (AJ633990)	98%	α-*proteobacteria*						1						
GQ301252	OTU-243	Unc α-*proteobacterium* (EF123300)	99%	α-*proteobacteria*									1			
GQ301258	OTU-244	Unc α-*proteobacterium* (EF220301)	99%	α-*proteobacteria*									1			
GQ301254	OTU-246	Unc α-*proteobacterium* (EF123378)	95%	α-*proteobacteria*									1			
GQ301263	OTU-247	Unc α-*proteobacterium* (EU246823)	96%	α-*proteobacteria*										1		
GQ301253	OTU-248	Unc α-*proteobacterium* (EF123311)	99%	α-*proteobacteria*										1		
GQ301300	OTU-249	Unc α-*proteobacterium* (EF123405)	99%	α-*proteobacteria*										1		
GQ301255	OTU-250	Unc α-*proteobacterium* (EF123405)	100%	α-*proteobacteria*										1		1
GQ301224	OTU-251	Unc α-*proteobacterium* (AJ633990)	98%	α-*proteobacteria*										1		
GQ301262	OTU-252	Unc α-*proteobacterium* (EF657852)	99%	α-*proteobacteria*										2		
GQ301264	OTU-253	Unc α-*proteobacterium* (EU636536)	100%	α-*proteobacteria*										2		
GQ301221	OTU-254	Unc α-*proteobacterium* (AJ633969)	99%	α-*proteobacteria*										2		
GQ301259	OTU-255	Unc α-*proteobacterium* (EF471686)	98%	α-*proteobacteria*										1		
GQ301261	OTU-256	Unc α-*proteobacterium* (EF630049)	100%	α-*proteobacteria*										1		
GQ301301	OTU-258	Unc α-*proteobacterium* (EF220574)	97%	α-*proteobacteria*								1				
GQ301227	OTU-260	Unc α-*proteobacterium* (AF236003)	94%	α-*proteobacteria*								1				
GQ301257	OTU-261	Unc α-*proteobacterium* (EF220223)	99%	α-*proteobacteria*							1					
GQ301241	OTU-262	Unc α-*proteobacterium* (DQ003179)	99%	α-*proteobacteria*							3					
GQ301231	OTU-263	Unc α-*proteobacterium* (AY162078)	90%	α-*proteobacteria*												1

GQ301260	OTU-264	Unc α-*proteobacterium* (EF629977)	95%	α-*proteobacteria*											1
GQ301234	OTU-265	Unc α-*proteobacterium* (AY710853)	97%	α-*proteobacteria*											1
GQ301230	OTU-266	Unc α-*proteobacterium* (AF473929)	97%	α-*proteobacteria*											1
GQ301216	OTU-267	Unc α-*proteobacterium* (AJ581348)	98%	α-*proteobacteria*										1	
GQ301240	OTU-269	Unc α-*proteobacterium* (AY942776)	97%	α-*proteobacteria*										1	
GQ301265	OTU-270	Unc α-*proteobacterium* (U78037)	99%	α-*proteobacteria*										1	
GQ301225	OTU-271	Unc α-*proteobacterium* (AM162572)	89%	α-*proteobacteria*										1	
GQ301223	OTU-272	Unc α-*proteobacterium* (AJ633989)	94%	α-*proteobacteria*										1	
GQ301245	OTU-273	Unc α-*proteobacterium* (DQ269055)	96%	α-*proteobacteria*										2	
GQ301219	OTU-274	Unc α-*proteobacterium* (AJ633964)	93%	α-*proteobacteria*										1	
GQ301232	OTU-275	Unc α-*proteobacterium* (AY580541)	93%	α-*proteobacteria*										1	
GQ301220	OTU-276	Unc α-*proteobacterium* (AJ633968)	91%	α-*proteobacteria*										1	
GQ301414	OTU-277	Bacterium Ellin5130 (AY234547)	99%	α-*proteobacteria*^					1						
GQ301422	OTU-279	Mucus bacterium (AY654746)	99%	α-*proteobacteria*^	5										
GQ301424	OTU-280	Mucus bacterium (AY654746)	99%	α-*proteobacteria*^			4								
GQ301522	OTU-281	Unc bacterium (AY242683)	94%	α-*proteobacteria*^	1										
GQ301497	OTU-282	Unc bacterium (AY373415)	93%	α-*proteobacteria*^	1										
GQ301510	OTU-283	Unc bacterium (DQ836762)	96%	α-*proteobacteria*^			1								
GQ301427	OTU-284	Unc bacterium (AY328849)	99%	α-*proteobacteria*^					1				1		
GQ301461	OTU-285	Unc bacterium (DQ416480)	95%	α-*proteobacteria*^									1		
GQ301486	OTU-286	Unc bacterium (EU491577)	97%	α-*proteobacteria*^										1	
GQ301436	OTU-287	Unc bacterium (DQ396355)	100%	α-*proteobacteria*^								1			
GQ301525	OTU-288	Unc bacterium (DQ985926)	99%	α-*proteobacteria*^										2	
GQ301519	OTU-289	Unc bacterium (DQ330954)	99%	α-*proteobacteria*^			1								
GQ301338	OTU-290	*Achromobacter* sp. (EU275167)	100%	β-*proteobacteria*		3	1				7	2			
GQ301342	OTU-291	*Delftia* sp. (EU019989)	99%	β-*proteobacteria*							3				
GQ301339	OTU-293	Unc β-*proteobacterium* (AY695723)	99%	β-*proteobacteria*					1		3		1	1	
GQ301341	OTU-294	Unc β-*proteobacterium* (EF061949)	99%	β-*proteobacteria*			3								
GQ301340	OTU-295	Unc β-*proteobacterium* (AY695723)	99%	β-*proteobacteria*			1								
GQ301493	OTU-297	Unc bacterium (AM697566)	99%	β-*proteobacteria*^		1									
GQ301369	OTU-298	*Acinetobacter* (EU078177)	99%	γ-*proteobacteria*										1	
GQ301370	OTU-299	*Alteromonas hispanica* (EU529840)	100%	γ-*proteobacteria*	3								1		1
GQ301371	OTU-300	*Escherichia* sp. (AM087500)	99%	γ-*proteobacteria*			1					1	2		1
GQ301394	OTU-302	*Methylophaga* (AM238553)	99%	γ-*proteobacteria*										1	
GQ301395	OTU-303	*Microbulbifer* sp. (AY862188)	92%	γ-*proteobacteria*			1								
GQ301396	OTU-304	*Moraxella osloensis* (EU400648)	99%	γ-*proteobacteria*									1		
GQ301397	OTU-305	*Neptuniibacter caesariensis* (AY136116)	97%	γ-*proteobacteria*	2										

GQ301398	OTU-306	*Photobacterium eurosenbergii* (AJ842345)	97%	γ-*proteobacteria*			1									
GQ301399	OTU-307	*Pseudoalteromonas* sp. (AF239705)	97%	γ-*proteobacteria*		1	1						1			
GQ301400	OTU-308	*Pseudomonas* sp. (AY014801)	99%	γ-*proteobacteria*			3	3	1		1		1		5	
GQ301401	OTU-309	*Salinivibrio* sp. (X95527)	99%	γ-*proteobacteria*								2				
GQ301402	OTU-310	*Serratia marcescens* (AY566180)	99%	γ-*proteobacteria*			1	3			1	2				
GQ301403	OTU-311	*Shewanella* sp. (AY573039)	100%	γ-*proteobacteria*			1									
GQ301404	OTU-312	*Shigella sonnei* (EU881979)	99%	γ-*proteobacteria*									3			1
GQ301405	OTU-313	*Spongiobacter nickelotolerans* (AB205011)	98%	γ-*proteobacteria*			1		2		1	1				
GQ301406	OTU-314	*Stenotrophomonas* sp. (AM402950)	100%	γ-*proteobacteria*	2	12	7	4	26	7	25	22			2	
GQ301407	OTU-315	*Thalassomonas* sp. (AY194066)	98%	γ-*proteobacteria*	9			1		1						
GQ301387	OTU-316	Unc γ-*proteobacterium* (AF228694)	93%	γ-*proteobacteria*	1											
GQ301391	OTU-319	Unc γ-*proteobacterium* (EF071131)	99%	γ-*proteobacteria*			1									
GQ301392	OTU-321	Unc γ-*proteobacterium* (X95229)	97%	γ-*proteobacteria*			1									
GQ301388	OTU-322	Unc γ-*proteobacterium* (AF424094)	97%	γ-*proteobacteria*			1									
GQ301386	OTU-323	Unc γ-*proteobacterium* (AJ240916)	99%	γ-*proteobacteria*			1									
GQ301380	OTU-324	Unc γ-*proteobacterium* (DQ351778)	98%	γ-*proteobacteria*				1								
GQ301389	OTU-325	Unc γ-*proteobacterium* (AY386332)	97%	γ-*proteobacteria*				1								
GQ301390	OTU-326	Unc γ-*proteobacterium* (AY499963)	97%	γ-*proteobacteria*				1								
GQ301393	OTU-327	Unc γ-*proteobacterium* (AY129103)	94%	γ-*proteobacteria*				1								
GQ301385	OTU-328	Unc γ-*proteobacterium* (AB015570)	100%	γ-*proteobacteria*				1								
GQ301377	OTU-329	Unc γ-*proteobacterium* (DQ167034)	96%	γ-*proteobacteria*						1						
GQ301375	OTU-331	Unc γ-*proteobacterium* (AY499917)	96%	γ-*proteobacteria*						1						
GQ301378	OTU-332	Unc γ-*proteobacterium* (DQ189758)	99%	γ-*proteobacteria*						1						
GQ301376	OTU-334	Unc γ-*proteobacterium* (AY515468)	90%	γ-*proteobacteria*						1						
GQ301383	OTU-335	Unc γ-*proteobacterium* (EF629798)	97%	γ-*proteobacteria*										2		1
GQ301381	OTU-336	Unc γ-*proteobacterium* (DQ857233)	91%	γ-*proteobacteria*								1				
GQ301382	OTU-338	Unc γ-*proteobacterium* (EF071131)	100%	γ-*proteobacteria*							1					
GQ301384	OTU-340	Unc γ-*proteobacterium* (EU005285)	94%	γ-*proteobacteria*												1
GQ301379	OTU-342	Unc γ-*proteobacterium* (DQ200403)	98%	γ-*proteobacteria*												1
GQ301374	OTU-343	Unc γ-*proteobacterium* (AM117932)	96%	γ-*proteobacteria*											1	
GQ301373	OTU-344	Unc γ-*proteobacterium* (AJ581349)	98%	γ-*proteobacteria*											1	
GQ301372	OTU-345	Unc γ-*proteobacterium* (AB116443)	96%	γ-*proteobacteria*											1	
GQ301408	OTU-346	Uncultured bacterium (AY529881)	93%	γ-*proteobacteria*		4	1									
GQ301409	OTU-347	Unc bacterium (AY700599)	96%	γ-*proteobacteria*			1									
GQ325718	OTU-348	*Vibrio coralliityticus* (AJ316167)	99%	γ-*proteobacteria*											10	

GQ301410	OTU-349	*Vibrio* sp. (DQ146982)	99%	γ-*proteobacteria*	5			1				1				
GQ325719	OTU-350	*Vibrio* sp. (EU372927)	99%	γ-*proteobacteria*												6
GQ301506	OTU-351	Unc bacterium (DQ163943)	99%	γ-*proteobacteria*^			1					1				
GQ301431	OTU-352	Unc bacterium (DQ163943)	99%	γ-*proteobacteria*^								1				
GQ301470	OTU-353	Unc bacterium (DQ990059)	99%	γ-*proteobacteria*^								1				
GQ301363	OTU-354	*Enhygromyxa* sp. (AB239010)	91%	δ-*proteobacteria*			1									
GQ301364	OTU-355	*Haliangium tepidum* (AB062751)	97%	δ-*proteobacteria*			3									
GQ301365	OTU-356	*Myxobacterium* (AB016469)	97%	δ-*proteobacteria*			1			2						
GQ301366	OTU-357	*Myxococcales* (EU437490)	94%	δ-*proteobacteria*												1
GQ301360	OTU-358	*Unc* δ-*proteobacterium* (AJ704691)	95%	δ-*proteobacteria*			1									
GQ301362	OTU-359	*Unc* δ-*proteobacterium* (DQ889925)	95%	δ-*proteobacteria*	2		1									
GQ301361	OTU-360	*Unc* δ-*proteobacterium* (AM039960)	97%	δ-*proteobacteria*			1									
GQ301359	OTU-361	*Unc* δ-*proteobacterium* (AB116392)	97%	δ-*proteobacteria*				1								
GQ301358	OTU-362	*Unc* δ-*proteobacterium* (EU050863)	94%	δ-*proteobacteria*					1							
GQ301356	OTU-363	*Unc* δ-*proteobacterium* (DQ109895)	95%	δ-*proteobacteria*									2			
GQ325720	OTU-365	*Unc* δ-*proteobacterium* (AY582894)	100%	δ-*proteobacteria*									1			
GQ301357	OTU-366	*Unc* δ-*proteobacterium* (EF414220)	98%	δ-*proteobacteria*										1		
GQ301355	OTU-367	*Unc* δ-*proteobacterium* (AY922068)	93%	δ-*proteobacteria*								1				
GQ301352	OTU-368	*Unc* δ-*proteobacterium* (AB116392)	97%	δ-*proteobacteria*											1	
GQ301353	OTU-369	*Unc* δ-*proteobacterium* (AJ704691)	95%	δ-*proteobacteria*											1	
GQ301354	OTU-370	*Unc* δ-*proteobacterium* (AY499979)	95%	δ-*proteobacteria*											1	

* Sequences were aligned to the closest relative over 700bp using BLAST (Zhang et al., 2000). The similarity was calculated with gaps not taken into account.

MC: *A. millepora* hosting clade C1 *Symbiodinium*

MD: *A. millepora* hosting clade D *Symbiodinium*

TC: *A. tenuis* hosting clade C1 *Symbiodinium*

TD: *A. tenuis* hosting clade D *Symbiodinium*

S-TC: *A. tenuis* hosting clade C *Symbiodinium* sampled in summer

S-TD: *A. tenuis* hosting clade C *Symbiodinium* sampled in summer

^ Sequence classification was based on the Ribosomal Database Project (RDP) alignments. Phylogenic affiliations included were >97% similar to clone sequences.