Exploring the function and behaviour of Natural Populations of Coral Reef Microbes

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Certificate of Original Authorship

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I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Jessica Tout
1/02/2016

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Publications

Peer reviewed journal articles arising directly from this thesis:

Chapter 1

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Chapter 2

Tout J, Jeffries TC, Petrou K, Tyson GW, Webster NS, Garren M, Stocker R, Ralph PJ, Seymour JR (2015). Chemotaxis by Natural Populations of Coral Reef Bacteria. ISME J doi: 10.1038/ismej.2014.261

Chapter 4

Tout J, Siboni N, Messer LF, Garren M, Stocker R, Webster NS, Ralph PJ and Seymour JR (2015). Increased seawater temperature increases the abundance and alters the structure of natural *Vibrio* populations associated with the coral Pocillopora damicornis. *Front. Microbiol.* 6:432. doi: 10.3389/fmicb.2015.00432

Chapter 5

Garren M, Son K, Raina JB, Rusconi R, Menolascina F, Shapiro OH, **Tout J**, Bourne DG, Seymour JR, Stocker R (2013). A bacterial pathogen uses dimethylsulfoniopropionate as a cue to target heat-stressed corals. ISME J 1-9, doi:10.1038/ismej.2013.210

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Garren M, Son K, **Tout J,** Seymour JR, Stocker R (2015). Temperature-induced behavioural switches in a bacterial coral pathogen. ISME J 1-10, doi:10.1038/ismej.2015.216

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Abstract

Microorganisms live in tight associations with corals, but the ecological interactions and microbial functions and behaviours underpinning these relationships are not yet fully understood. The goal of this thesis is to define coral-microbe interactions by exploring how the composition, behaviour and function of microbial communities vary throughout a coral reef and how increasing sea water temperatures can affect coral-microbial relationships. As a first step to achieving this aim, In Chapter 1 we used metagenomics to characterise patterns in microbial composition and metabolic capacity across different niches, including coral-associated and non-coral associated microenvironments, on Heron Island, the Great Barrier Reef (GBR). We found that the composition and metabolic potential of coral reef bacteria is highly heterogeneous across a coral reef ecosystem, with a shift from an oligotroph-dominated community (e.g. SAR11, *Prochlorococcus, Synechococcus*) in the open water and sandy substrate niches, to a community characterised by an increased frequency of copiotrophic bacteria (e.g. *Vibrio, Pseudoalteromonas, Alteromonas*) in the coral seawater niches. Among the major functional patterns observed were significant increases in genes associated with bacterial motility and chemotaxis in samples associated with the surfaces of coral colonies. The observation of increased motility and chemotaxis near to coral surfaces is notable given previous evidence that these phenotypes may be involved in coral disease processes. The research presented in this chapter was published in Microbial Ecology (2014 67 (3): 540-552)

To investigate these patterns in chemotaxis further we next (Chapter 2) directly examined the potential ecological role of chemotaxis among coral-associated bacteria, by using laboratory based and *in situ* chemotaxis assays to test levels of chemotaxis among natural communities of coral reef microbes. We examined the behavioural responses towards several chemoattractants known to be released by corals and their symbiotic dinoflagelletes including amino acids, carbohydrates, ammonium chloride, and dimethylsulfonopropionate (DMSP). Using these approaches we found that bacteria associated with the surfaces of the corals

exhibited high levels of chemotaxis, particularly towards DMSP and several amino acids. Levels of chemotaxis by coral-associated bacteria were consistently higher than those demonstrated by non-coral associated bacteria. This work was published in the ISME Journal (doi: 10.1038/ismej.2014.261)

We next extended the *in situ* chemotaxis assays to examine the chemotactic behaviour of bacteria associated with other important coral reef organisms, sponges. These results redefine the sponge-symbiont acquisition paradigm whereby we show for that bacteria use chemotaxis to locate their sponge host on a coral reef. This work is in preparation for submission to the ISME Journal.

After defining some of the functions and behaviours involved in coral reef microbiology, we next examined how these processes may shift under changing environmental conditions, associated with climate change. To determine how environmental variability, specifically thermal stress, influences bacterial community composition, behaviour and metabolic capacity, manipulation experiments were conducted using the coral *Pocillopora damicornis*.

To investigate the dynamics of coral-associated vibrios under heat stress, in Chapter 4 we used Vibrio-specific amplicon sequencing approaches and qPCR to quantify shifts in the abundance and composition of natural populations of *Vibrio*, with a specific focus on the putative coral pathogen *V. coralliilyticus*. These experiments revealed that increasing seawater temperatures can favour the proliferation of potential coral pathogens among a natural mixed microbial community. This work has been published in Frontiers in Microbiology (6:432. doi: 10.3389/fmicb.2015.00432).

In Chapter 5, we decided to explore the entire coral-associated community by using metagenomics and metatranscriptomics to investigate how the phylogeny and function of coral associated microbes shift resulting from increasing seawater temperatures. We found a dramatic shift in the community from *Endozoicomonaceae* being dominant in the control corals, while there was an appearance of the vibrios under increasing sea water temperatures in line with our

findings from chapter 4. We also observed functional shifts that involved an up-regulation of chemotaxis and motility genes at higher temperatures and were shown to be affiliated with vibrios, a genus which contains several putative coral pathogens.

Taken together our data demonstrate that coral reef bacterial communities are highly dynamic and that key groups of copiotrophic bacteria have the capacity to use behaviours such as chemotaxis to use nutrient gradients to potentially locate and colonize benthic host animals including corals and sponges. Increasing seawater temperatures causes dramatic changes in the coral-associated bacterial community, allowing for the proliferation of potential coral pathogens and increased expression of behavioural phenotypes that may promote successful infection of corals.

Introduction

Corals and their symbiotic partners

Scleractinian corals are benthic marine animals within the class Anthozoa and phylum Cnidaria and are characterised by the production of an exoskeleton composed of calcium carbonate, which forms the foundation of coral reef ecosystems (Hutchings et al., 2008). Coral reefs are the largest living structures on Earth and among these, the Great Barrier Reef (GBR) covers 2000 km and comprises over 3000 individual reefs (De'ath et al., 2012). The GBR is economically valuable, generating approximately $6.1 billion USD per year from tourism alone (Hoegh-Guldberg et al., 2004). In addition they support the majority of fisheries worldwide, with one third of all fish species dependant on coral reefs at some stage in their life cycle (Jones et al. 2004).

In 1883 Karl Brandt discovered that scleractinian corals host a dynamic community of endosymbionts including dinoflagellate algae (Brandt, 1883). These algae were first cultured in the 1950s (Rosenberg et al., 2007), which resulted in the discovery of a new genus known as *Symbiodinium* (Taylor, 1993), now commonly referred to as 'zooxanthellae'. A variety of genetically diverse *Symbiodinium* clades inhabit scleractinian corals, and each influence the corals growth and resilience to stress in different ways, for example by differential contributions to energy budgets or thermal tolerance (Hill, Ulstrup & Ralph 2009; Berkelmans and Van Oppen 2006). The coral host can obtain energy autotrophically when supported by the chemical products released from the *Symbiodinium* during photosynthesis. This photo-symbiosis provides the coral host with up to 90% of its essential nutrients, including oxygen, photosynthetically fixed carbon, amino acids and also helps to synthesise calcium carbonate. In turn, the coral host supplies their resident *Symbiodinium* with a habitat and access to CO_2, ammonium, phosphorus and iron as nutrients for photosynthesis and metabolism (reviewed in Bourne and Webster 2013). This symbiotic relationship allows both the coral host and *Symbiodinium* to survive in otherwise oligotrophic waters (Allers et al. 2008; Houlbreque & Ferrier-Pages 2009). The coral host maximises this symbiotic relationship by limiting the availability of organic nitrogen which not only limits *Symbiodinium* growth but

also increases photosynthesis (Muscatine et al., 1989; Bythell, 1990; Falkowski et al., 1993). Approximately 20%-40% of the excess photosynthate released to the coral is subsequently used to produce coral mucus, which is released in copious amounts by many coral species (Davies, 1984; Crossland, 1987; Edmunds and Davies, 1989). Notably, individual coral polyps are also capable of heterotrophic filter feeding, by using their nematocysts or stinging cells to capture various planktonic organisms including algae, larvae, *Symbiodinium* and bacteria (Wild et al. 2004).

In addition to *Symbiodinium*, corals host a dynamic community of other microorganisms including bacteria, viruses, protists, fungi, archaea and algae (Rosenberg et al., 2007; Vega-Thurber et al., 2009). Microbial communities are now considered a functionally important part of a tripartite coral symbiosis, which includes the cnidarian host, the *Symbiodinium* (Rohwer et al. 2002) and the associated microorganisms, which co-exist within a mutualistic microcosm referred to as the 'coral holobiont' (Rohwer 2010; Rosenberg et al. 2007).

Microbes in Corals

Microorganisms comprise by far the largest diversity of marine organisms (Webster and Hill, 2007) and are responsible for a number of essential ecosystem services, including fundamental roles in the marine carbon and nitrogen cycles (Webster & Hill 2007; Falkowski et al., 2008; Bourne and Webster, 2013). While more than 100 million microbes occur per cm^2 of a healthy corals surface (Paul et al., 1986) and identifying their taxonomy is well established, the functional diversity of these microbes and their roles in regulating coral health and function have only recently become a greater focus of coral reef research.

Who and where are they?

Coral colonies host diverse bacterial communities that are phylogenetically distinct, more active and more abundant than bacterial populations in adjacent seawater (Ducklow & Mitchell 1979; Paul et al., 1986; Rath et al., 1993; Ritchie

et al. 1996; Rohwer et al. 2001; Rosenberg et al. 2007; Weinbauer et al., 2010). For example, bacterial populations inhabiting the coral surface microlayer (CSM) are 100 to 1000-fold more abundant than bacterial populations in the surrounding seawater (Rosenberg et al. 2007).

The coral mucocyte cells within the CSM generate mucus which is a polysaccharide-protein (Sharon & Rosenberg 2008) rich in carbohydrates such as glucose, fucose, arabinose, mannose and galactose (Meikle, Richards & Yellolees 1998). Bacteria can actively metabolise these carbohydrates and proteins (Paul et al., 1986), which represent important resources for bacteria growing in otherwise oligotrophic waters (Brown & Bythall 2005; Shnit-Orland & Kushmaro 2009). It has therefore been proposed that coral mucus largely controls the composition of the coral-associated bacteria (Ritchie, 2006). In addition to the CSM, heterotrophic bacteria colonise various other 'coral niches', including the skeleton, the tissue layer and the gastrodermal cavity (Rosenberg et al. 2007). There is evidence that the microbial communities within these different niches are phylogenetically different (Sweet et al., 2011), while it has also been noted that different coral morphologies may play a role in structuring bacterial diversity (Sunagawa et al., 2010).

Despite relatively few species being examined in detail, some studies have shown that coral-bacteria are species-specific regardless of geographic location (Rohwer et al., 2002; Chen et al., 2011), with microbial assemblages on specific coral species conserved across different regions of the global ocean. In contrast, other research has showed that geographic location, rather than coral species determines the microbial community composition, highlighting the influence that environmental conditions can have on microbial associations (Littman et al., 2009; Barott et al., 2011). Additionally, seasonal variations in the microbial community have been identified in some coral species (Hong et al., 2009; Chen et al., 2011). Taken together these patterns reveal the highly dynamic nature of coral microbial assemblages, which may form important ecological roles within coral reef ecosystems.

The functional roles of coral-associated microbes

Microbes can have various functional roles on coral reefs. Over large scales, heterotrophic bacteria play fundamental roles in biogeochemical cycling on coral reefs by remineralizing dissolved and particulate organic substrates released from the reef (Sorokin 1978; Wild et al., 2005; Webster & Hill 2007; Falkowski et al., 2008; Raina et al., 2009a; Bourne and Webster, 2013). While at smaller scales, microbes play vital roles in regulating the health and physiology of their coral host (Rosenberg et al., 2007; Bourne et al., 2009) by performing important chemical processes, such as nitrogen fixation, within the coral microenvironment, which ultimately sustain the symbiotic relationship between the coral host and *Symbiodinium*, (Lema et al., 2012; Santos et al., 2014; Radecker et al., 2015). Microbes can form a variety of relationships with corals including:

(i) Commensalism, where they have no measurable effect on the coral.

(ii) Mutualism, where they provide a 'beneficial' role to corals. Microbes can play this role by recycling nutrients near to the coral host (Sorokin, 1973; Bourne and Webster 2013), or protecting their coral host against pathogens through the production of antibiotic compounds (Rohwer et al., 2002; Resheft et al., 2006; Ritchie, 2006; Rosenberg et al., 2007). Additionally, the 'Coral Probiotic Hypothesis' proposes that corals harbour a diverse and dynamic microbial consortia that assists the coral to adapt to environmental changes (Reshef et al., 2006) and enhances its resilience to stress by promoting the growth of helpful microbes and controlling the growth of pathogenic microbes (Klaus et al., 2005).

(iii) Pathogenicity, where microbes cause disease, particularly during times when the coral is stressed due to changes in environmental conditions (Rohwer 2010).

Corals and disease

The role of bacteria as coral pathogens has dominated coral-microbiology research due to coral disease and bleaching contributing to the destruction of coral

reefs globally (Richardson 1998; Frias-Lopez et al 2002; Klaus et al 2005; Rosenberg 2007; Vega-Thurber et al 2009; Mitta et al 2011). During the past three decades close to thirty coral diseases have been described (Bourne et al., 2009) however, confirming the pathogen responsible for disease through fulfilment of Koch's postulates has proven difficult, because the majority of marine microorganisms are not culturable (Jannasch and Jones. 1959; Hoppe, 1976, Bianchi and Giuliano, 1996). Furthermore, often it appears that some diseases are caused by a consortium of microbes, rather than a single species (Sato et al., 200?). Currently, seven diseases responsible for coral mortality have been linked to specific microbial species through the fulfilment of Koch's postulates, and include: plague (Richardson et al., 1998), white syndrome – causing bleaching and lysis (Kushmaro et al., 1996; Ben-Haim et al., 2003; Rosenberg and Falkovitz 2004; Sussman et al., 2008), white band (Ritchie and Smith 1998), white plague (Thompson et al., 2001), white pox (Patterson et al., 2002), aspergillosis (Richardson et al., 2001) and yellow band (Cervino et al., 2008), which have all led to mass mortalities and significant losses of corals world-wide (Bourne et al., 2009). Furthermore, it has been suggested that changes in environmental conditions such as an increase in Sea Surface Temperatures (SST) can stress corals (Mouchka et al., 2010; Ruiz-Morenol et al., 2012), resulting in higher susceptibility to infection by opportunistic pathogens (Bourne et al., 2009; Mouchka et al., 2010) that may also become more abundant or virulent under elevated temperatures (Vega-Thurber et al., 2009; Vezzulli et al., 2010; Kimes et al., 2011; de O Santos et al., 2011).

To date, half of the identified coral pathogens belong to the *Vibrionaceae* family of bacteria (Rosenberg et al., 2007). These include *V. owensii* which causes white syndrome in *Monitpora* corals (Ushijima et al., 2012), *V. charchariae* (synonym for *V. harveyi*), which is responsible for white band disease II in *Acropora cervicornis* (Gil et al., 2006; Sweet et al., 2014), and a consortium of *Vibrios* have been implicated in yellow band disease (Cervino et al., 2008). The majority of research investigating the roles of Vibrios in coral health and disease has been conducted in the laboratory using cultured isolates (Kushmaro et al. 1998; Banin et al. 2000; Ben-Haim et al, 2002, 2003; Koren and Rosenberg 2006; Vidal-dupiol

2011a; Garren et al. 2014; Rubio-Portillo et al. 2014; Garren et al., 2014), however it is also important to consider how these bacteria interact with natural mixed populations of microbes in more natural scenarios laboratory conditions may not be reflective of the complex dynamic ecosystems that occurs on coral reefs.

Bacterial bleaching of corals was first reported by Kushmaro and colleagues (1996) following extensive bleaching of *Oculina patagonica* in the Mediterranean Sea. Bleaching of *O. patagonica* was found to be caused by infection by the bacterium *Vibrio shiloi*. Notably, infection only occurred at temperatures above 25°C, and both elevated temperatures and *V. shiloi* had to be present for coral bleaching to occur (Kushmaro et al 1996; Rosenberg et al 2009). Three virulence factors are required for *V. shiloi* to successfully attach itself to coral and disrupt the coral-*Symbiodinium* symbiosis (Rosenberg et al 2009; Rohwer 2010). The first involves adhesion to the coral surface through chemotaxis to receptors in the mucosal layer, the second is the production of the Toxin P, which is produced by *V. shiloi* and inhibits *Symbiodinium* photosynthesis, leading to cell lysis, expulsion and consequent coral bleaching, and the third is the production of superoxide dismutase (SOD), a defence against Reactive Oxygen Species (ROS) (Rosenberg et al 2007).

Similarly, *Vibrio coralliilyticus* causes tissue loss and bacterial bleaching of the coral *Pocillopora damicornis* (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b; Meron et al., 2009; Garren et al., 2014). The mechanisms underpinning infection by *V. coralliilyticus* are similar to those used by *V. shiloi* whereby there is an increased infection rate at elevated SST (Toren et al., 1998; Ben-Haim and Rosenberg 2002; Ben-Haim et al., 2003b), *V. coralliilyticus* exhibits enhanced motility and chemotaxis under elevated SST to locate the coral host (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b) and coral tissue is damaged through the production of an extracellular zinc metalloproteinase (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b). While *V. shiloi* and *V. coralliilyticus* cannot infect healthy coral, it is hypothesised that elevated temperature disrupts the production of antibiotics by the resident bacterial community and simultaneously increases the virulence of opportunistic pathogens, which infect

the coral when it is most vulnerable to infection (Rosenberg et al 2009). Furthermore, natural communities of *Vibrio* species become more prevalent at higher seawater temperatures, with this shift preceding the onset of coral bleaching in the coral *Acropora millepora* on the GBR. Elevated SST's cause changes in the resident microbial community, increasing both the virulence of pathogenic bacteria and their ability to infect weakened corals (Rosenberg et al 2007; Bourne et al 2008; Rohwer 2010).

Environmental Change and Threats to Corals

Environmental perturbations including climate change, disease, increases in anthropogenic pollution, cyclones and crown-of-thorns starfish (COTS) can be a significant stress for corals, which already survive within a narrow range of optimum environmental conditions. In particular, temperature-dependent bleaching events are serious threats to coral reefs. Bleaching is a stress response to changes in environmental conditions and results in the coral expelling their endosymbiotic *Symbiodinium* and as a consequence losing their pigmentation and up to 63% of their nutrition (Glynn, 1991; Hoegh-Guldberg et al., 2007). If elevated temperatures are sustained for long enough coral mortality can result, ultimately leading to severe and widespread declines in coral cover (Berkelmans et al., 2004). *Symbiodinium* in corals, respond to thermal stress in similar ways to terrestrial plants, whereby inhibition of photosythnesis occurs (Schreiber and Bilger 1987; Walker et al., 1992), resulting in temperature-induced oxidative stress (Lesser, 1997) leading to cellular damage (Hoegh-Guldberg 1999). The effect of increasing seawater temperature on the coral host and the *Symbiodinium* is well studied in coral biology, yet the effect of temperature stress on coral-associated microbes is less well known and to date, the majority of studies have been limited to only a few cultured bacterial isolates under laboratory conditions (Kushmaro et al., 1998; Toren et al., 1998; Banin et al., 2001; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b; Garren et al., 2014), while molecular studies have also been limited (Vega-Thurber et al. 2009; Littman et al. 2011). Environmental stress is thought to increase coral mucus production within the CSM, which will concurrently increase the density and activity of the resident

microbial communities (Segal & Ducklow 1982; Ritchie et al. 1996; Ceh et al., 2011). Additionally, the community composition and functional potential of coral-associated microbial communities shift in response to changing environmental conditions (Vega-Thurber et al., 2009). However, the ecological interactions between the various components of the coral holobiont (coral, *Symbiodinium*, microbial community) under changing environmental conditions are poorly characterised despite these processes and interactions being fundamental to understanding coral health. The application of molecular techniques to coral microbiology has further revolutionised our understanding of these complex and dynamic associations between corals and microbes (Rohwer et al., 2002; Rosenberg et al., 2007; Ceh et al., 2001, 2012).

Molecular Analysis of Coral Microbial Communities

Until the 1980's, standard microbial cultivation techniques were used to examine coral-associated bacteria. However, because more than 99% of marine microorganisms are unable to be cultured, this approach provided a very restricted and often inaccurate view of coral microbiology. 16S rRNA gene sequencing was first applied to coral microbiology in 2001 by Rohwer and colleagues, who discovered a variety of microbial groups previously not known to associate with corals. The subsequent widespread application of 16S rRNA gene sequencing approaches (Bourne and Webster, 2013) revolutionised our understanding of coral-associated microbial diversity, including patterns of host and geographic specificity, and insights into disease dynamics (Frias-Lopez et al., 2002). However, while 16S rRNA gene sequencing provided coral microbiologists with a universal culture-independent method for identifying bacterial diversity, it did not offer any insight into microbial function or physiology (Handelsman, 2004), as many closely related bacterial species carry out different metabolic functions within the microbial community. The introduction of metagenomics to coral microbiology in 2007 by Wegley and colleagues, provided the first insights into both the phylogenetic structure and potential functional roles of coral-associated bacteria.

Metagenomics allows for the identification of the taxonomic composition and functional potential of microbial communities from an environmental sample without the need to amplify genomic DNA. The first coral-microbial metagenomics study proposed functional roles for the various microbes associated with the coral *Porites astreoides* including bacteria, bactriophage, endolithic fungi and eukaryotic viruses, in particular highlighting new nutrient cycles within the holobiont (Wegley et al., 2007). Metagenomics has also been applied over different spatio-temporal scales, providing an insight into how microbial function and community composition changes throughout space and time. On the scale of entire coral reef ecosystems, metagenomics has been used to examine shifts in the structure and function of microbial communities collected from seawater across island chains and coral atolls (Dinsdale et al., 2008; Bruce et al., 2012), indicating that the level of anthropogenic impacts on a reef can affect the function and composition of coral associated microbial assemblages.

To date, two studies have used metagenomics to track changes in coral-microbial communities during times of environmental stress (Vega-Thurber et al., 2009; Littman et al., 2011). Changes in the microbial community associated with *Acropora millepora* on Magnetic Island (GBR) were monitored using metagenomics during a naturally occurring temperature-dependent bleaching event. The metabolism of the microbial community associated with bleached corals displayed a higher tendency for heterotrophy and had a higher proportion of genes associated with virulence factors relative to healthy corals (Littman et al., 2011). The metagenomic approach becomes even more powerful when combined with manipulative experiments to assess the effects of environmental changes on microbial functional potential. Vega-Thurber et al., (2009) was the first to use metagenomics to show that coral-associated bacterial communities respond to changes in abiotic factors that can cause stress to the coral holobiont, by examining the effects of increased temperature, elevated nutrients and dissolved organic carbon, and reduced pH (Vega-Thurber et al., 2009). This study demonstrated that of these environmental variables, increases in temperature had the greatest effect on the microbial community associated with the coral species *Porites compressa,* which shifted to a more pathogenic state, including a the

occurrence of a significantly higher proportion of pathogenic *Vibrio* species. This work indicated that environmental factors can increase the potential for opportunistic pathogens to invade the coral through an increased abundance of genes associated with virulence, motility and chemotaxis (e.g. flagella assembly, flagella motor rotator and gliding motility genes) (Vega-Thurber et al. 2009).

The observations of elevated motility and chemotaxis genes within the heat-stressed samples in the Vega-Thurber study is notable, because there is evidence that these phenotypes may be particularly important within coral-microbe associations. Laboratory studies using cultured isolates highlight that chemotaxis may be an important component in the pathogenicity of coral disease causing bacteria; as demonstrated by the enhanced ability of the pathogens *V. shiloi* and *V. coralliilyticus* to locate and cause infection of corals when motile and chemotactic (Koren & Rosenberg 2006; Rosenberg et al 2007; Mitta et al. 2011b, Garren et al., 2014). However, despite this evidence from some coral pathogens, the behavioural mechanisms by which bacteria use to locate their coral hosts remains relatively unknown, and the potential role of chemotaxis in mediating coral-microbe interactions will be a major focus of this thesis.

Chemotaxis allows bacteria to move in response to chemical stimuli (Lux and Shi, 2004; Seymour et al. 2008) and as early as 1976, it has been suggested that marine bacteria may be attracted to coral and *Symbiodinium* exudates (Chet and Mitchel 1976). Beyond being involved in pathogenicity, chemotaxis may assist bacteria in finding specific coral niches, such as the CSM and despite the mounting evidence that marine bacteria can be highly chemotactic (Banin et al. 2001; Koren & Rosenberg 2006; Rosenberg et al 2007; Stocker et al 2008; Seymour et al 2009, 2010; Stocker and Seymour et al. 2012), no studies have yet explored the chemotactic abilities of mixed communities of coral-associated bacteria. To date, all studies reporting chemotaxis have been conducted in the laboratory under controlled conditions using cultured isolates of bacteria and whether or not chemotaxis is used by natural coral-reef microbes to locate their coral host remains unknown.

While metagenomic research has enabled coral microbiologists to identify the potential importance of functional genes, such as those involved in chemotaxis and motility, among coral-associated microbial communities under a variety of environmental conditions the activity or expression of these genes is relatively unexplored. Metatranscriptomic approaches are better suited for assessing the influence of changing environmental conditions on functional variation and regulation within the transcriptionally active fraction of microbial communities at a particular time (Moran et al. 2013). Metatranscriptomics will provide for a better assessment of the active gene transcription in the microbial community by generating a better understanding of how the microbial community function and activity responds to changing environmental conditions (Moran et al., 2009), and this approach will be applied to understand the effects of rising seawater temperature on coral-microbe interactions in this thesis.

This Study

Despite significant research progress during the last two decades, the ecology of coral reef microbes, including the mechanisms involved in establishing coral-bacteria relationships and the functional response of the microbial communities' to environmental change are still not fully understood. The over-arching goal of this research is to define coral-microbe interactions by exploring how the composition, behaviour and function of microbial communities vary throughout a coral reef and how increasing sea water temperatures can affect coral-microbial relationships. This will be achieved by coupling ecogenomic approaches with novel in situ microfluidic based experimental systems and manipulative experiments. The specific aims of this thesis are to:

1. Determine how the composition and functional potential of microbial communities shift between different microniches distributed across a single coral reef ecosystem.
2. Examine the extent to which natural populations of coral reef-associated bacterial communities exhibit chemotactic behaviour in response to

chemicals released by corals and determine the identities of the microbes responding to specific chemical cues.

3. Determine whether chemotaxis is involved in the establishment of symbiotic relationships between microbes and other benthic reef animals (sponges)
4. Investigate how increasing seawater temperature affects the composition of natural populations of coral-associated microorganisms and understand the effects on putative coral pathogens
5. Couple manipulative experiments and ecogenomic approaches to determine how changing environmental conditions (e.g. rising seawater temperatures) will influence the functional capacity of coral associated microbial assemblages.

The successful achievement of these aims will provide a new understanding of naturally occurring coral-reef microbial communities, in particular the microbial seascape of coral reefs: how bacteria locate corals and sponges on coral reefs and what impact increasing SST will have on microbial communities and their important ecological functions.

Chapter 1:

Variability in microbial community composition and function between different niches within a coral reef

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Abstract

To explore how microbial community composition and function varies within a coral reef ecosystem, we performed metagenomic sequencing of seawater from four niches across Heron Island Reef, within the Great Barrier Reef. Metagenomes were sequenced from seawater samples associated with: (i) the surface of the coral species *Acropora palifera* (ii) the surface of the coral species *Acropora aspera* (iii) the sandy substrate within the reef lagoon and (iv) open water, outside of the reef crest. Microbial composition and metabolic function differed substantially between the four niches. The taxonomic profile showed a clear shift from an oligotroph dominated community (*e.g.*, SAR11, *Prochlorococcus, Synechococcus*) in the open water and sandy substrate niches, to a community characterised by an increased frequency of copiotrophic bacteria (*e.g., Vibrio, Pseudoalteromonas, Alteromonas*) in the coral seawater niches. The metabolic potential of the four microbial assemblages also displayed significant differences, with the open water and sandy substrate niches dominated by genes associated with core house-keeping processes such as amino acid, carbohydrate and protein metabolism as well as DNA and RNA synthesis and metabolism. In contrast, the coral-surface seawater metagenomes had an enhanced frequency of genes associated with dynamic processes including motility and chemotaxis, regulation and cell signalling. These findings demonstrate that the composition and function of microbial communities are highly variable between niches within coral reef ecosystems, and that coral reefs host heterogeneous microbial communities that are likely shaped by habitat structure, presence of animal hosts and local biogeochemical conditions.

Key words: *Metagenomics, Coral Reefs, Niche, Microbes*

Introduction

Microbes play a fundamental role in determining the health and ecology of coral reefs (Krediet et al., 2013; Rosenberg et al., 2007). At the scale of an entire reef, microbes drive chemical cycling processes that ultimately control the productivity and biogeochemical function of the reef ecosystem (Di Salvo and Gundersen, 1971; Sorokin, 1971; Webster and Hill, 2007; Raina et al., 2010; Stocker and Seymour, 2012). At finer scales, microbes form both mutualistic and pathogenic relationships with a variety of benthic organisms including corals, sponges, clams, ascidians and macroalgae (Wahl, 1995; Jensen et al., 1996; Webster et al., 2001; Rohwer et al., 2002; Webster et al., 2012a). At the scale of the individual coral colony the abundance, taxonomic composition and metabolic potential of bacterial communities inhabiting the gastrodermal cavity, skeleton and surface microlayer of a coral differ substantially (Sweet et al., 2011). However, as a consequence of the complex physical and chemical nature of coral reef ecosystems, we are only just beginning to unravel the spatio-temporal dynamics of coral reef microbial communities (Moriarty et al., 1985; Paul et al., 1986; Seymour et al., 2005; Sweet et al., 2011).

In recent years, molecular microbiological techniques have provided important insights into patterns in coral reef microbial diversity and function, enhancing our understanding of how microbes influence overall coral reef ecosystem function and stability (Dinsdale et al., 2008; Littman et al., 2009; Webster et al., 2010a). Coral colonies have been shown to host diverse and species-specific microbial communities (Rohwer et al., 2001, 2002; Littman et al., 2009) that are typically phylogenetically distinct from those in adjacent waters (Rohwer et al., 2001, 2002; Frias-Lopez et al., 2002; Kellogg, 2004; Rosenberg et al., 2007). Clear dissimilarities between the microbial assemblages associated with healthy corals, diseased corals, and dead coral surfaces have also been demonstrated by 16S rRNA gene sequencing (Frias-Lopez et al., 2002). Furthermore, microbes associated with other benthic organisms on coral reefs have shown similar patterns of host-specificity. For instance, sponges harbour diverse and abundant microbial communities, which generally exhibit species specificity regardless of geography (Hentschel et al., 2002; Webster et al., 2012b).

Characterisation of the local spatio-temporal dynamics and regional biogeographical patterns of coral reef microbial communities has recently been assessed using molecular tools such as 16S rRNA sequencing. For instance, Littman et al. (2009) revealed that patterns of species specificity among the microbial communities inhabiting three species of Great Barrier Reef (GBR) *Acropora* corals were not conserved according to geographic location. More recently, an examination of the impact of tidal cycles on the spatio-temporal patterns of coral reef bacteria on Heron Island demonstrated relatively homogenous microbial assemblages between sample sites (Sweet et al., 2011). However, these observations are in contrast to previous work demonstrating significant differences in microbial community composition between sample sites on coral reefs at Lizard Island (GBR), Magnetic Island (GBR), and Tobago (Caribbean) (Moriarty et al., 1985; Seymour et al., 2005; Guppy et al., 2006). Nelson et al. (2011) examined the spatio-temporal variations throughout Paopao Bay (French Polynesia) by measuring changes in the community composition between waters from the bay, the reef and the open ocean, revealing distinctly different phylogenetic features of the bacterial communities between sites.

The microbial communities inhabiting healthy, diseased and bleached *Acroporas* from American Samoa and the Great Barrier Reef have also recently been shown to demonstrate compositional shifts under natural bleaching and disease events, with the abundance of *Vibrio* bacteria increasing during bleaching (Bourne et al., 2008; Wilson et al., 2012). While these studies have begun to reveal the complexities of microbial community ecology on coral reefs, our understanding of how microbial function changes over a coral reef ecosystem is less well developed.

Metagenomic analyses have recently begun to provide insights into the diversity and functional roles of microbes within coral reef ecosystems. For instance, metagenomics has provided snapshots of the structure and function of the microbial community associated with the coral *Porites astreoides* (Wegley et al., 2007), revealing that endolithic algae can be key players in the microbial community and drive important chemical processes such as nitrogen fixation within the coral holobiont. Metagenomics has also been used in manipulative experiments to

demonstrate shifts in the composition and metabolic potential of coral-associated microbes under changing environmental conditions such as increased temperature, elevated nutrients, DOC and decreased pH (Vega-Thurber et al., 2009). This work by Vega-Thurber et al. (2009) demonstrated that environmental stress led to a shift from a mutualistic to potentially pathogenic microbial community, as indicated by a substantial increase in genes involved in virulence, stress resistance, chemotaxis, and motility.

On a broader scale, metagenomics has been used to assess the effects of natural temperature variability during a bleaching event on Magnetic Island (GBR), revealing a shift in the community composition of microbes associated with a transition from healthy to bleached health state of the coral *Acropora millepora* (Littman et al., 2011). This study also reported a shift in metabolism from autotrophy in healthy corals to heterotrophy in bleached corals and a higher proportion of virulence factors in the microbial community associated with bleached corals. Furthermore, metagenomics has shown clear shifts in taxonomic composition and metabolic potential between four coral atolls in the Northern Line Islands (central Pacific ocean (Dinsdale et al., 2008) and the Abrolhos Bank coral reefs off the coast of Brazil (south-western Atlantic ocean) (Bruce et al., 2012).

The coral reef metagenomic studies performed to date have collectively enhanced our understanding of coral reef microbiology by describing patterns in microbial structure and function in specific coral species (Wegley et al., 2007; Vega-Thurber et al., 2009; Littman et al., 2011; Bruce et al., 2012), across different coral reef ecosystems (Dinsdale et al., 2008; Bruce et al., 2012) and under changing environmental conditions and stress levels (Vega-Thurber et al., 2009; Littman et al., 2011. However, we still have little insight into how the composition and function of microbial assemblages varies within a single reef ecosystem. Consequently, we lack a full appreciation of the way in which within reef heterogeneity influences microbial survival, adaptation or niche exploitation. Here, we present a metagenomic study aimed at determining microbial function and composition across different niches within a single reef ecosystem, as a first step towards dissecting how within-reef variability influences the associated microbial communities.

Methods

Niches and water collection

Sampling was conducted on Heron Island in the Capricorn Bunker Group on the southern Great Barrier Reef (23°26'31.20”S, 151°54'50.40”E) during July 2011. Four different seawater niches were selected for sampling based on different reef features (Gourlay, 1996), and included: i) the sandy substrate, ii) coral surfaces in the lagoon, iii) coral surfaces in the reef-crest and iv) the open water. Sample collection occurred on an incoming tide at the same time of day over the course of four consecutive days, approximating the same point within the tidal cycle, where one sample was collected per day.

Coral surface seawater samples were collected adjacent to the surface of a colony of *Acropora palifera* situated in the lagoon (23°26’41”S, 151°54’47”E) and a colony of *Acropora aspera* located on the reef-crest (23°26’41”S, 151°54’47”E) (Supplementary Information Fig. S 1.1). These samples are henceforth referred to as lagoon-coral and reef crest-coral, respectively. These coral species were chosen because they represent the dominant species in the respective zones of the Heron Island Reef (Wild et al., 2004). Coral-associated seawater samples were collected by placing the mouth of a sterile 10 L Schott bottle immediately over (within 1 cm) the surface of the coral.

The sandy substrate seawater sample was collected immediately above (1 cm) the sandy substrate within the Heron Island lagoon (23°26’36”S, 151°54’47”E), in a location where the water depth was 40 cm and no corals were present within a radius of 10 m (Supplementary Information Fig. S 1.1). Finally, an open water sample was taken from the water surface at a point approximately 3 km outside of Heron Island’s north-western fore-reef slope (23°24’58”S, 151°53’12”E), where the water depth was 40 m (Supplementary Information Fig. S 1.1).

Microbial cell counts

Four 500 µL water samples were collected from each of the niches, fixed with glutaraldehyde (1% final concentration) and frozen in liquid nitrogen before being stored at -80 °C. Prior to flow cytometry (FCM), samples were quick-thawed, stained with SYBR Green I [1:10,000] (Invitrogen Molecular Probes USA) and 1 µm diameter fluorescent microspheres (Invitrogen Molecular Probes USA) were added as an internal reference (Marie et al., 1997). Samples were analysed using a Becton Dickinson LSR II flow cytometer (BD Biosciences) and bacterioplankton populations were discriminated according to SYBR Green fluorescence and side-scatter (Marie et al., 1997). FCM data was analysed using Cell-Quest Pro software (BD Biosciences) and bacterioplankton abundances were compared between niches using a 1-way Analysis of Variance (ANOVA), where normal distribution of data was ensured using Levene's homogeneity of variance test. All analyses were performed using Minitab statistical software (Version 15.1.0.0 2006, Pennsylvania, USA).

DNA collection and extraction

In each of the four niches, 10 L of seawater was collected and immediately returned to the Heron Island Research Station laboratories, where it was filtered onto 0.2 µm polycarbonate membrane filters (Millepore) within 10 min of collection. The filters were frozen at -80 °C until DNA extraction was conducted using the MO BIO PowerWater DNA isolation kit (Carlsbad, California, USA) according to the manufacturer's instructions. Genomic DNA concentrations were measured using a Qubit 2.0 fluorometer (Invitrogen).

Sequencing and Bioinformatics

A shotgun metagenomic library was prepared and sequenced for each of the four samples using the 454 GS-FLX pyrosequencing platform (Roche) at the Ramaciotti Centre for Gene Function Analysis at the University of New South Wales. Sequences were subsequently analysed using the *Meta Genome Rapid Annotation using*

Subsystems Technology (MGRAST; Version 3 pipeline (Overbeek et al., 2005; Meyer et al., 2008)). Quality control was performed by removing reads with >10 ambiguous bases per read and de-replicating artificial duplicates in which the first 50bp of the read were identical (Table 1.1). Within MG-RAST, metabolic assignments were annotated to the SEED subsystems database (Overbeek et al., 2005) and taxonomic identification was determined based on the top BLAST hits to the SEED taxonomy. The SEED is organised into three hierarchical levels for metabolism and six levels for taxonomy (Overbeek et al., 2005; Meyer et al., 2008; Edwards et al., 2010; Pfister et al., 2010; Poroyko et al., 2010; Delmont et al., 2011; Jeffries et al., 2011, 2012; Seymour et al., 2012; Smith et al., 2012). Matches with a E-value of <0.05 were considered significant with a minimum alignment of 50 bp (Vega-Thurber et al., 2009; Edwards et al., 2010; Pfister et al., 2010; Poroyko et al., 2010; Delmont et al., 2011; Jeffries et al., 2011, 2012; Seymour et al., 2012; Smith et al., 2012). All data was normalised to sequencing effort by dividing by the total number of hits. The metagenomes can be accessed through MG-Rast (http://metagenomics.anl.gov/) under the project numbers 4483104.3 (lagoon-coral), 4483105.3 (reef crest-coral), 4483106.3 (sandy substrate), and 4483107.3 (open water).

Taxonomic and metabolic reconstructions generated using MG-RAST were imported into the Statistical Analysis of Metagenomic Profiles (STAMP) package to test for statistically significant differences between the 4 metagenomes. Fisher's exact test was used to determine significant differences between samples with a Benjamini FDR multiple test correction applied (Benjamini and Hochberg, 1995). We used Fisher's exact test (Agresti, 1990; Rivals et al., 2007) to identify the most significantly different categories (taxonomic or metabolic categories) between two samples, as suggested in the STAMP user manual (Parks and Beiko, 2010) and as has been recommended (Rivals et al., 2007; Parks and Beiko, 2010) and routinely applied in this fashion (Polato et al., 2011; Jeffries et al., 2012; Seymour et al., 2012; Shi et al., 2012; Sharp et al., 2012). Hence, all quoted q-values represent corrected values (equating to q), with only values <0.05 reported (Parks and Beiko, 2010). Confidence intervals (95%) were determined using the Newcombe–Wilson method. Using this approach we compared the relative frequency of taxonomic groups at the

class level for archaea, at the order level for eukaryota, the genus level for bacteria, and the species level for viruses. The different taxonomic levels were chosen for the domains to display the taxonomic differences between the niches at the highest resolution without compromising the abundance of sequences per taxa generated at each level. The metabolic capacity of the communities was examined using the SEED level 1, 2 and 3 groupings (Parks and Beiko, 2010).

Multivariate statistical software (PRIMER v6) was used to measure the degree of similarity between metagenomes (Clarke and Gourley, 2006). Data was square-root transformed and the Bray-Curtis similarity was calculated between samples. SIMPER analysis (Clarke. 1993) was used to identify the phylogenetic groups and metabolic categories contributing most to the dissimilarity between the metagenomes.

Results

Metagenomic sequencing was used to assess variability in microbial community composition and functional potential across four different reef niches on Heron Island. FCM counts showed highest bacterial abundance in the open water niche ($P<0.05$), with bacterial abundance decreasing progressively from the reef crest-coral to lagoon-coral and sandy substrate niche (Table 1.1). There were between 103900 to 233026 sequences across the metagenomes, with average read lengths of between 449 to 457bp (Table 1.1). When annotated and compared to the SEED database, the metagenomic data revealed significant changes in microbial community composition and metabolic potential across the different coral reef niches.

Taxonomy

By comparing sequences in the four metagenomes to the SEED database, between 42 - 59% of sequences could be matched to known phylogenies (Table 1.1), which is a level of phylogenetic assignment consistent with previous ocean metagenomes (Hewson et al., 2009). All samples were dominated by *Bacteria,* with between 84 -

89% of sequences matching bacteria (Table 1.1), and of these *Proteobacteria* made up an average of 57% of bacterial matches. Matches to *Eukaryota* comprised between 5.2-9% of sequences, 3.3-6.6% of sequences matched *Viruses* and 0.8-1.2% of sequences matched *Archaea.*

Table 1.1 Environmental data and metagenome statistics for the four Heron Island niches

Meta data	**Sandy substrate**	**Lagoon-coral**	**Reef Crest-coral**	**Open Water**
DNA Sequences	233,026	201,910	182, 182	103, 900
Sequences fail QC	23, 899	20, 691	18, 818	10, 573
Mean sequence length of DNA base pairs (Post QC)	457	450	449	454
Known annotated proteins % (SEED)	54	42	50	59
% of matches to Bacteria	88.4 (58.3% to Proteobacteria)	84 (56.3% to Proteobacteria)	84.6 (60.9% to Proteobacteria)	89.5% (55% to Proteobacteria)
% of matches to Eukaryota	5.2	9	7.3	5.2
% of matches to Viruses	4.4	5.3	6.6	3.3
% of matches to Archaea	1.2	0.9	0.8	1.1
Bulk counts of bacteria per mL +/- SD	9.4×10^4 +/- 1.2×10^4	3.7×10^5 +/- 6.7×10^4	1.0×10^6 +/- 1.6×10^5	3.5×10^6 +/- 4.5×10^5
Sea water temperature °C	19.7	19.7	19.6	19.7

Shifts in microbial community composition were observed between the four niches (Fig. 1.1) and were found to be statistically significant ($q<0.05$) using Fisher's exact test (Fig. 1.2). SAR11 was the most abundant bacterial group in all samples (Fig 1.1). Statistically significant shifts in the relative abundance of SAR11 were observed between different Heron Island Reef niches ($q<0.05$), where SAR11 was

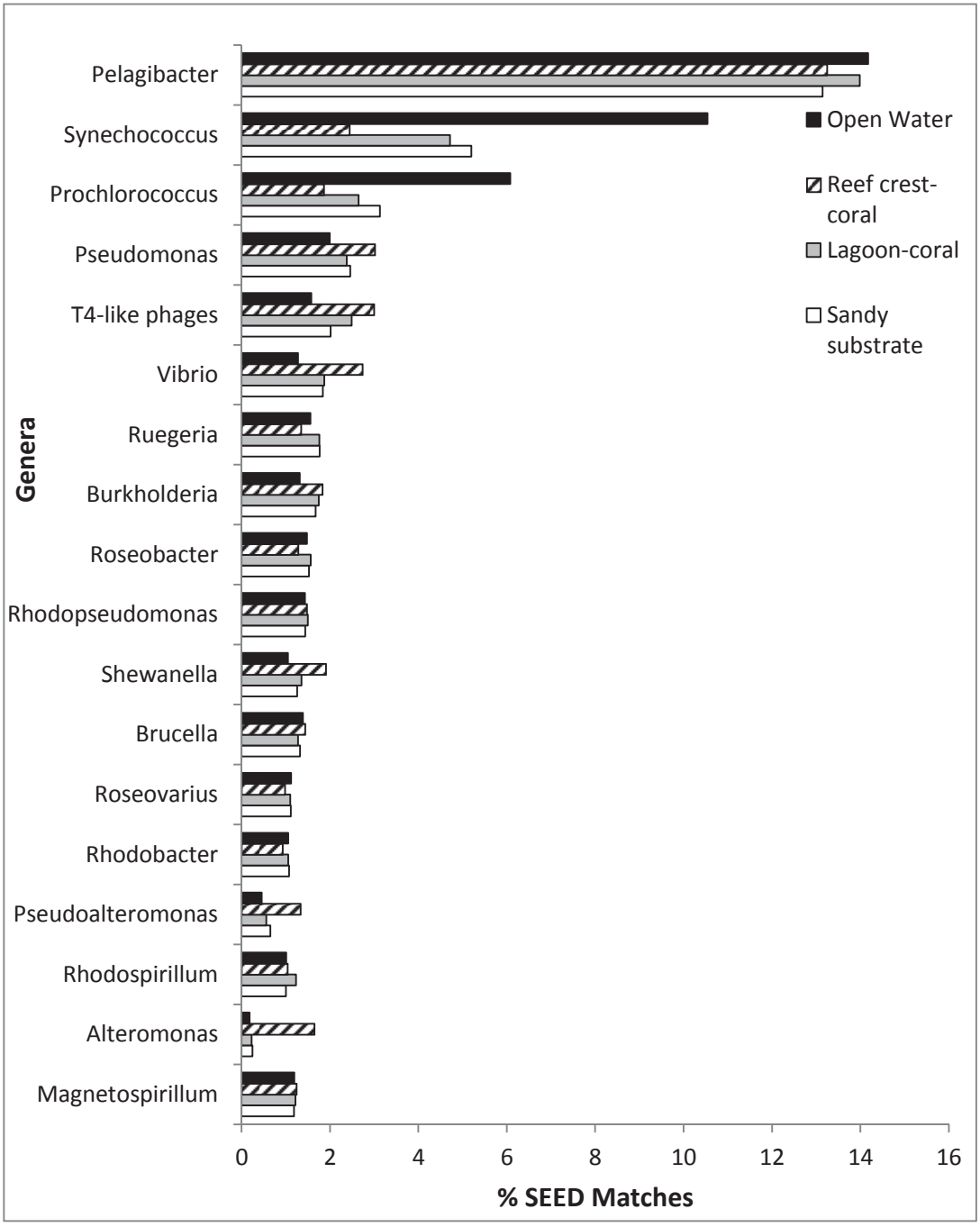


Figure 1.1 Top 18 microbial genera (relative % of microbial SEED matches) in each seawater niche metagenome: sandy substrate, lagoon-coral, reef crest-coral and open water (ordered according to frequency of occurrence in each niche)

significantly less abundant in the reef-crest coral niche than in all other niches. However, SIMPER analysis revealed that this clade was not a significant driver of the overall variation in community composition (Fig. 1.1, Supplementary Table S1.1). Other microbes including the marine cyanobacteria *Synechococcus* and *Prochlorococcus*, were also abundant in all niches sampled, but made up a

significantly greater proportion of the open water community than within the other niches ($q<0.05$) (Figs. 1.1-1.2). In contrast to SAR11, SIMPER analysis revealed that *Synechococcus* and *Prochlorococcus* were responsible for driving statistically significant differences in community composition between the sea water niches and were the most discriminating genera of the non-coral niches (open water and sandy substrate) (Supplementary Table S1.1). *Synechococcus* and *Prochlorococcus* together accounted for 5.7% of the dissimilarity between metagenomes.

While SAR 11, *Prochlorococcus* and *Synechococcus* were generally the most abundant bacteria in all niches, several bacteria occurred in increased frequency in the reef crest-coral niche than in the open water or sandy substrate niches ($q<0.05$) (Figs. 1.1-1.2). These included *Pseudomonas, Vibrio, Shewanella, Pseudoalteromonas, Mycobacterium* and *Alteromonas* (Figs. 1.1-1.2) with all but *Shewanella* and *Mycobacterium* significantly more abundant in the lagoon-coral niche than the open water niche (Fig 1.1, Supplementary Table S1.2).

SIMPER analysis revealed that these genera were responsible for driving statistically significant differences in community composition between the seawater samples (Fig. 1.2, Supplementary Table S1.1), with *Alteromonas, Mycobacterium* and *Vibrio* identified as discriminating genera of the coral-associated (reef crest-coral and lagoon-coral) niches. Combined, these groups accounted for 3.6% of the dissimilarity between metagenomes (Supplementary Table S1.1).

Archaea comprised between 0.8-1.2% of sequences across all niches. Hits to the Euryarchaeota phylum (1.45%) comprised the greatest number of sequences across all metagenomes, followed by Thaumarchaeota (0.53%) and Crenarchaeota (0.18%) (Supplementary Figure S1.2). Sequence matches to an unclassified Thaumarchaeota class were higher than matches to any other class found across the metagenomes (Supplementary Figure S1.2) and were significantly more abundant in the sandy substrate niche than any other niche (Fisher's exact test, $q<0.05$) (Supplementary Figure S1.2). The Euryarchaeota populations were mainly comprised of the following classes: Methanomicrobia which was less abundant in the open water niche than any other niche ($q<0.05$), Halobacteria showed no differences in abundance across the niches, Thermoprotei which was more abundant in the open water than in the lagoon-coral niche only ($q<0.05$), Archaeoglobi was more

abundant in the coral niches than the non-coral niches ($q<0.05$) and Methanopyri which was more abundant in the reef-crest than any other niche (Supplementary Figure S1.2).

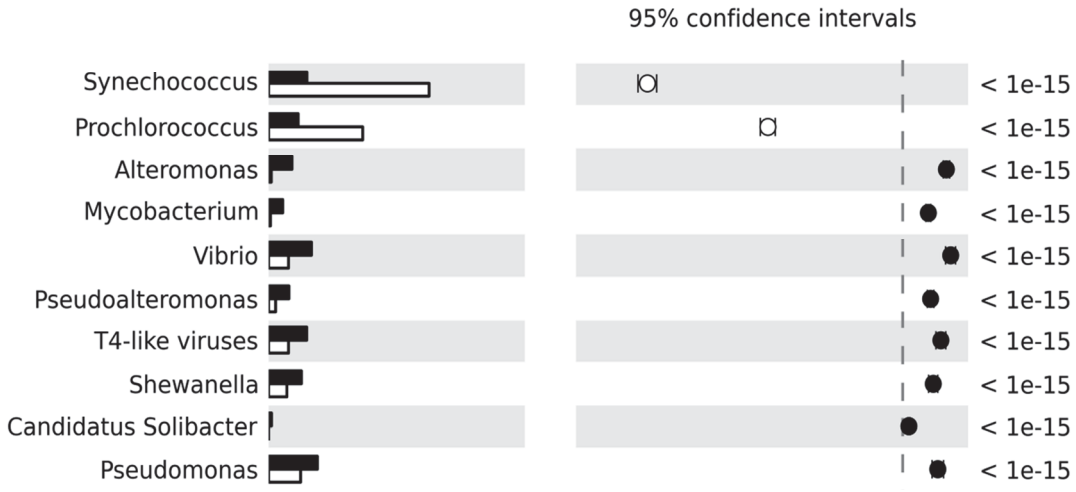


Figure 1.2 Fisher's exact test was used to statistically test for significant differences in the relative representation of microbial genera between the reef-crest coral community and open water community. The top 10 most significant differences are displayed here out of 149 genera that were found to be significantly different between the reef crest-coral seawater niche and the open water niche. A) Groups over-represented in the reef crest-coral seawater community (black) correspond to positive differences between proportions. Groups over-represented in the open seawater community (white) correspond to negative differences between proportions

We also observed an increase in sequences matching T4-like phages in the two coral associated metagenomes compared to the non-coral niches (open water and sandy substrate niches) (Figs. 1.1-1.2, Supplementary Table S1.2 and S1.3). Phages belonging to T4-like phages included *Prochlorococcus* phages P-SSM2, P-SSM4 and *Synechococcus* phage S-PM2, which dominated all samples, comprising between 95-98% of all T4-like phage sequences retrieved at each niche (Supplementary Figure S1.3). *Prochlorococcus* and *Synechococcus* phages were significantly more abundant ($q<0.05$) in the reef crest-coral and lagoon-coral niches than the open water and SIMPER analysis revealed that these groups contributed to significant differences between coral (reef crest-coral and lagoon-coral) and non-coral (open water and sandy substrate) niches.

While the sequences matching *Brassicales* from Eukaryota represented only 0.45% of all sequences, this group comprised the greatest number of eukaryotic sequences across all niches (Supplementary Figure S1.4) and was more abundant in the reef crest-coral niche than the sandy-substrate niche ($q<0.05$). Matches to other less abundant eukaryota such as *Scleractinia* were significantly more abundant in the coral niches than the non-coral niches (Supplementary Figure S1.4) ($q<0.05$). *Chlorellales* was more abundant in the reef crest-coral niche ($q<0.05$), *Actiniaria* was more abundant in the lagoon-coral niche ($q<0.05$) and *Cypriniformes* was more abundant in the sandy-substrate than in any of the other niches ($q<0.05$) (Supplementary Figure S1.4).

Function

Sequence matches to genes fundamental to basic microbial function, often referred to as "core" or "house-keeping" genes, comprised the most abundant hits in all metagenomes. These included genes involved in carbohydrate, amino acid, protein, RNA and DNA metabolism, respiration, and nucleoside synthesis (Fig. 1.3). Despite their predominance across all niches, SIMPER analysis revealed that variations in the occurrence of these house-keeping genes did not drive significant differences between metagenomes (Figs. 1.3-1.4, Supplementary Table S1.4), although Fisher's exact test revealed that many of these gene groups were significantly more abundant in the open-water metagenome compared to the reef crest-coral metagenome ($q<0.05$) (Fig. 1.4). In contrast, metabolic categories with comparatively lower abundance were responsible for the greatest variation in functional potential between the microbial communities. In particular, genes associated with bacterial motility and chemotaxis, regulation and cell signalling varied significantly between niches ($q<0.05$) (Figs. 1.4a-4d) and SIMPER analysis revealed genes associated with these categories were responsible for significant separation between the four metagenomes (Supplementary Table S1.4). These gene groups were also significantly more abundant within the reef-crest-coral metagenome than at any other niche ($q<0.05$) (Fig. 1.4).

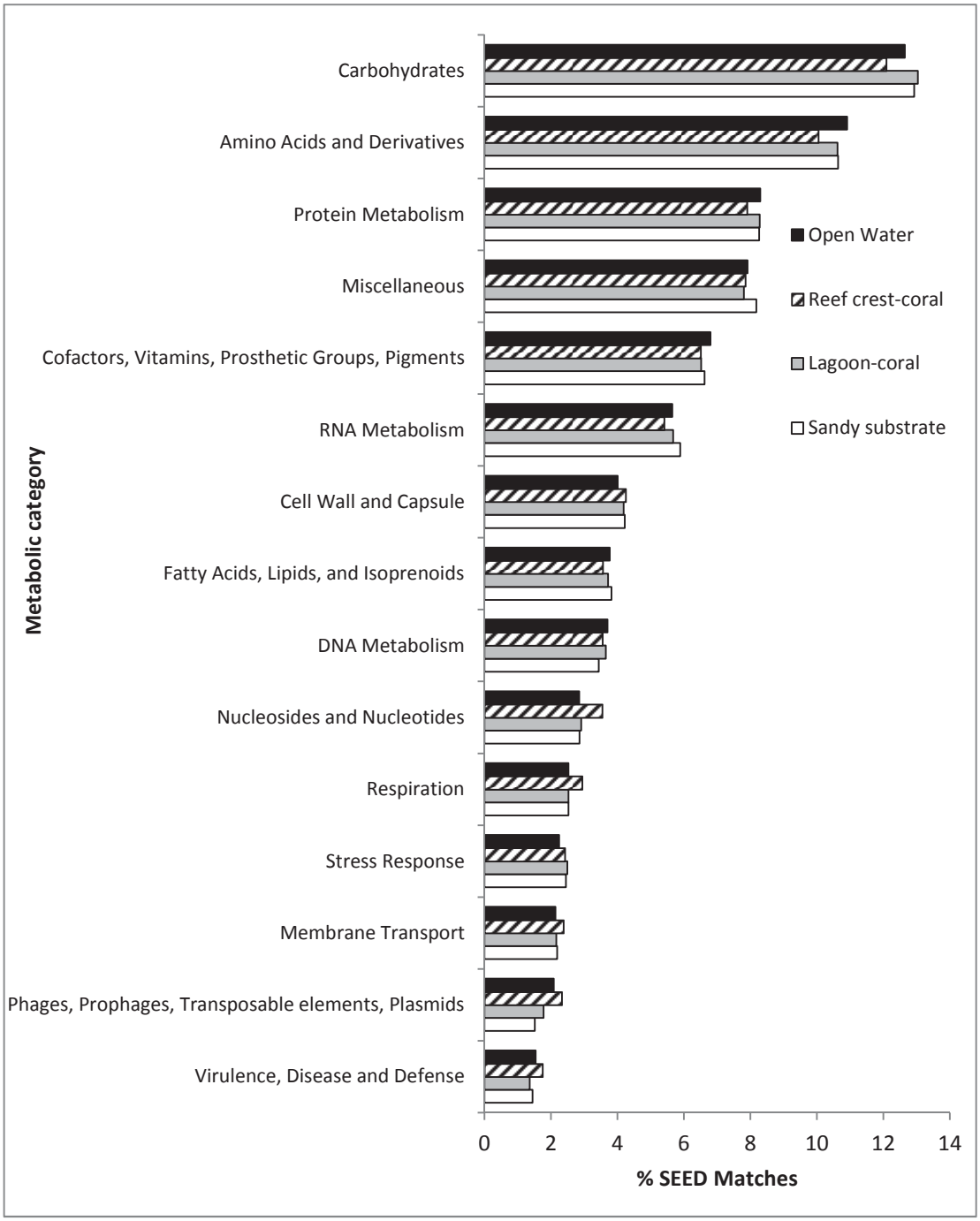


Figure 1.3 Frequency distribution (relative % of microbial SEED matches) of top 15 microbial metabolic functions in each seawater niche metagenome: sandy substrate, lagoon-coral, reef crest-coral and open water (ordered according to frequency of occurrence in each niche)

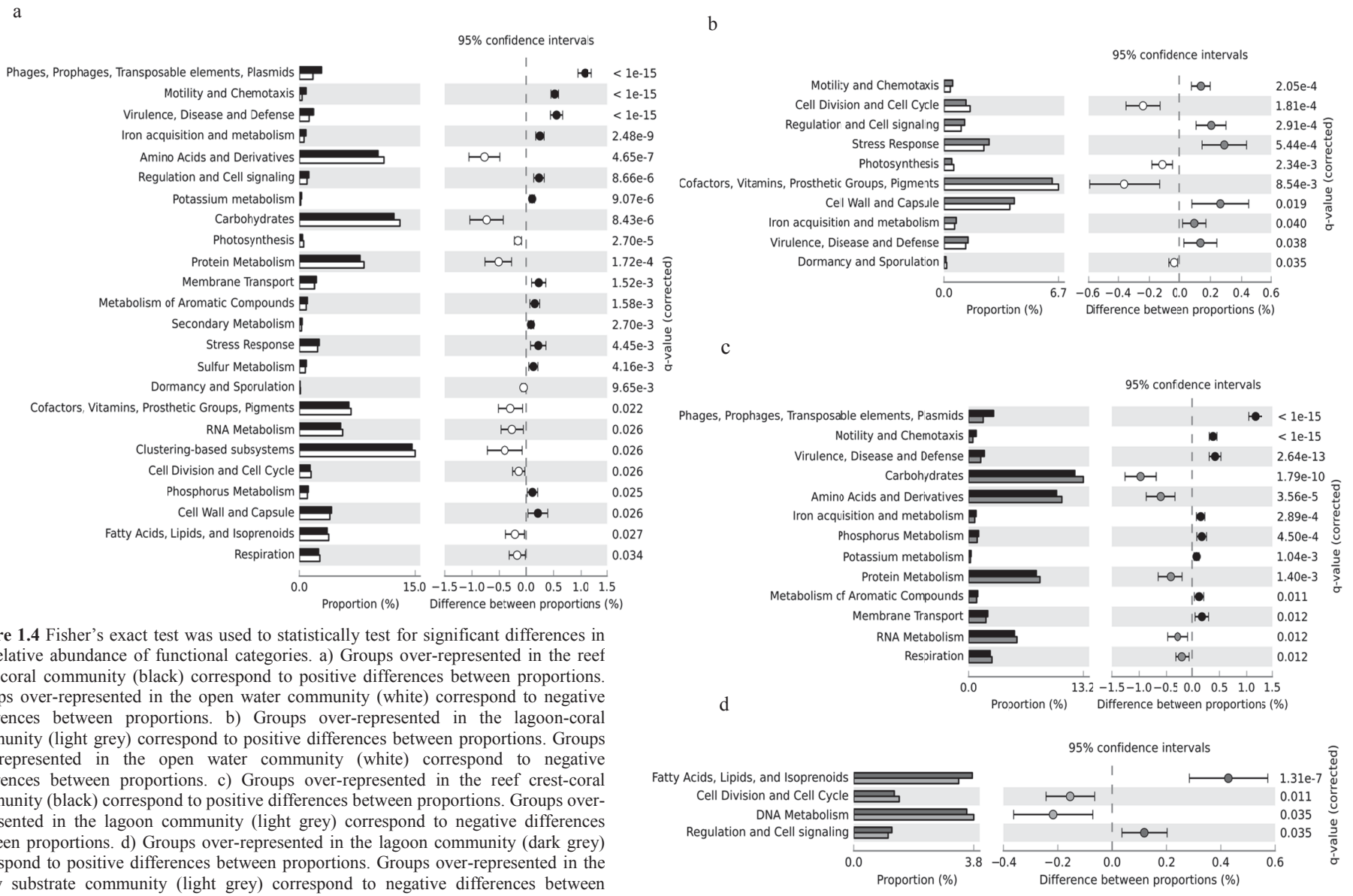


Figure 1.4 Fisher's exact test was used to statistically test for significant differences in the relative abundance of functional categories. a) Groups over-represented in the reef crest-coral community (black) correspond to positive differences between proportions. Groups over-represented in the open water community (white) correspond to negative differences between proportions. b) Groups over-represented in the lagoon-coral community (light grey) correspond to positive differences between proportions. Groups over-represented in the open water community (white) correspond to negative differences between proportions. c) Groups over-represented in the reef crest-coral community (black) correspond to positive differences between proportions. Groups over-represented in the lagoon community (light grey) correspond to negative differences between proportions. d) Groups over-represented in the lagoon community (dark grey) correspond to positive differences between proportions. Groups over-represented in the sandy substrate community (light grey) correspond to negative differences between proportions

Phage associated genes (in particular genes affiliated with phage tail proteins, phage integration and phage excision) also varied significantly between niches ($q<0.05$) (Figs. 1.4a-4d). Overall, the reef crest-coral niche contained a greater abundance of phage-associated genes than the open water niche ($q<0.05$) (Fig. 1.4a) and the lagoon-coral metagenome had a significantly higher proportion of genes associated with this category than the sandy substrate ($q<0.05$) (Fig. 1.4d).

Several other metabolic categories typically related to more specialised processes, also occurred in significantly higher proportions in the reef crest-coral niche metagenome than in the open water niche. These included membrane transport, iron acquisition and metabolism, potassium metabolism, metabolism of aromatic compounds, cell wall and cell capsule synthesis and stress response (Fig. 1.4a). In contrast, other metabolic categories including photosynthesis and respiration were more abundant in the open water niche ($q<0.05$) (Fig. 1.4a-b). Photosynthetic genes, specifically genes associated with light-harvesting complexes and photosystem I, were most abundant in the open water niche ($q<0.05$), whereas genes associated with photosystem II were higher in the reef crest-coral niche ($q<0.05$).

Discussion

In this study, metagenomic sequencing revealed substantial variability between the four metagenomes despite the close proximity of the samples (<500 m between the sandy substrate, lagoon-coral and reef-crest coral). These data are indicative of substantial heterogeneity in microbial community composition and metabolic potential across different niches within a single coral reef ecosystem. Previous research has used metagenomics to reveal large changes in microbial community composition and function between different coral reefs (Dinsdale et al., 2008; Littman et al., 2009). However, while 16S rRNA based approaches have shown that microbial community composition can shift within reef systems (Moriarty et al, 1985; Seymour et al., 2005; Guppy and Bythell, 2006; Nelson et al., 2011), patterns in microbial functional potential across different niches within a single coral reef ecosystem had not previously been documented.

Taxonomy

Microbial taxonomy varied significantly between the four niches, with differences often related to the presence or absence of corals. We also found evidence that the trophic strategies of coral-associated microbes were distinct to the non-coral microbial communites. It has previously been demonstrated that marine microbes broadly fall into two trophic categories, defined as copiotrophic and oligotrophic organisms (Lauro et al., 2009). Oligotrophs are generally highly abundant, but slow growing microbes that dominate within environments characterised by stable and low nutrient conditions (Lauro et al., 2009). Conversely, copiotrophs are microbes that are generally less abundant, but capable of bursts of rapid growth in response to intermittent pulses of high nutrient concentrations and often live in association with plant and animal hosts (Lauro et al., 2009). Our data are indicative of a partitioning of these life strategies between the different coral reef niches sampled.

Consistent with most marine metagenomic studies, sequence matches to the classic marine oligotroph *Candidatus pelagibacter* (SAR11) dominated all samples (Morris et al., 2002; Brown et al., 2012). However, the relative importance of SAR11 differed between samples, with sequence matches to SAR11 significantly less abundant in the reef crest-coral niche. Similarly, the two common cyanobacterial genera *Synechococcus* and *Prochlorococcus*, which are also typical of oligotrophic open water niches, were significantly more abundant in the non-coral associated niches (open water and sandy substrate). It has previously been demonstrated that extracts from 100 coral species, including 8 *Acropora* species, produce anti-microbial compounds against *Synechococcus* spp. (Koh, 1997). While our finding that *Synechococcus* sequences occur at lower frequency in the coral-associated metagenomes is consistent with this observation, the simultaneous decrease in sequences matching the other dominant oceanic microbes (SAR 11 and *Prochlorococcus*) indicates that more general environmental differences between the open water and coral-associated niches may be responsible.

While oligotrophs dominated the non-coral niches, we observed an increase in the numbers of sequences matching copiotrophic organisms in the coral-associated

samples. This included statistically significant increases in the relative importance of copiotrophic bacteria including *Pseudomonas, Vibrio, Shewanella, Pseudoalteromonas, Mycobacterium*, and *Alteromonas*. These increases in copiotrophic organisms are consistent with the higher concentrations of organic material typically found near the surfaces of corals compared to the surrounding open water (Sorokin, 1973; Meikle et al., 1988; Wild et al., 2004; 2004b; 2010).

Corals provide a niche enriched in dissolved and particulate organic material (Sorokin, 1973; Raina et al., 2010; Paul et al., 1986; Wild et al., 2004; 2004b, 2010). For instance, coral mucus and the exudates of *Symbiodinium* spp. are rich in organic compounds, including amino acids, sugars and dimethylsulfoniopropionate (DMSP) (Von Holy and Von Holt, 1968; Meikle et al., 1988; Wild et al., 2005; 2010; Raina et al., 2009; Bourne et al., 2013), meaning that concentrations of these compounds can be two to four orders of magnitude higher near the coral surface than in the surrounding seawater (Broadbent et al., 2004). Diffusion of this dissolved organic material and shedding of coral mucus into the water immediately overlaying coral surfaces is likely to produce a localised plume of organic material available to bacteria in the water column, and the elevated occurrence of copiotrophic bacteria in the coral associated metagenomes is consistent with this.

Notably, many of the copiotrophic bacteria identified here can represent either beneficial bacteria for the corals or potential pathogens. Several of the microbial genera that were over-represented in the reef crest-coral niche have been identified as potential coral pathogens. For instance, *Vibrios* have been implicated in several coral diseases. *V. shiloi* and *V. coralliilyticus* have been shown to be involved in coral bleaching (Banin et al., 2001; Rosenberg et al., 2007; Kimes et al., 2011), *Vibrio owensii* was found to cause *Montipora* white syndrome (Ushijima et al., 2012) and a consortium of *Vibrios* are believed to be involved in Yellow Band Disease (Cervino et al., 2008; Ushijima et al., 2012; Sato et al., 2009, 2010; Bourne et al., 2011). However, while the roles of *V. shiloi* and *V. corallilyticus* in bleaching has been confirmed by several studies (Banin et al., 2001; Rosenberg et al., 2007; Kimes et al., 2011) it is still not clear whether other *Vibrios* found in association with diseased corals (Cervino et al., 2008; Ushijima et al., 2012; Sato et al., 2009, 2010; Bourne et

al., 2011) are the disease causing agent, or opportunistic colonisers that most efficiently exploit already compromised corals (Raina et al., 2009). In addition to potential pathogens, some of the bacterial genera over-represented in the reef crest-coral metagenome may have beneficial effects for the coral. Among these, members of the *Pseudomonas* genus have been shown to inhibit the growth of potential pathogens when isolated from the soft coral *Sinularia polydactyla* (Salasia and Lammler, 2008) and members of the *Pseudoalteromonas* genus have been shown to inhibit the settlement of the coral pathogen *V. shiloi* (Nissimov et al., 2009).

Recently, it has been demonstrated that the 16S rRNA sequences of coral-associated Archaea on Heron Island, the Gulf of Eilat and the Virgin islands are more than 97% similar (Siboni et al., 2008). Our results demonstrate that at a finer-scale, the distribution and diversity of Archaea is not uniform across Heron Island reef. *Archaea* only comprised a maximum of 1.2% of all sequences, but this is comparable to the relative abundance of archaeal sequences found in other coral reef environments (Bruce et al., 2012).

The most dominant Archaeal class across all metagenomes was an unclassified representative from the Thaumarchaeota phylum which was found to be in higher abundance in the sandy substrate niche. Thaumarchaeota have recently been detected in benthic coral reef organisms such as marine sponges (Fan et al., 2012) and tropical and temperate ascidians (Erwin et al., 2013). There is some evidence that the Thaumarchaeota may be involved in ammonia oxidisation within sponges and ascidians (Fan et al., 2012; Erwin et al., 2013). There is less evidence for ecological associations between Thaumarchaeota and corals and the distributional dynamics of this group across different coral reef niches is previously unknown. Our results indicate that the relative importance of Thaumarchaeota is higher in the sandy-substrate, lagoon-coral and reef crest-coral niches than within open water, indicating that this group may have greater ecological importance in coral reef ecosystems than the surrounding open waters.

Euryarchaeota were collectively the most dominant Archaea phylum across all metagenomes. Previously, euryarchaeota have been shown to be present in warm

shallow waters associated with coral reefs (Kellogg, 2004) and can comprise a significant proportion of both the coral and sponge-associated prokaryotic communities (Kellogg, 2004; Wegley et al., 2004; Webster et al., 2012a; Fan et al., 2012). Our results demonstrate for the first time the distribution of euryarchaeota throughout a single reef ecosystem where the only class which showed a homogeneous distribution across all niches was Halobacteria, with the remaining eight archaeal classes being found in different abundances throughout the niches. Notably, Archaeoglobi was the only class to be more abundant in the coral-niches than the non-coral niches and Methanopyri was more abundant in the reef-crest niche than any other niche. Among the Crenarchaeota, which are often the dominant Archaeal group in marine samples, Thermoprotei was the only class represented and accounted for only 0.03% of sequences. This phylum includes members that have previously been shown to form associations with coral (Siboni et al., 2008). While our results have shown that the distribution and abundances of crenarchaeota and euryarchaeota throughout Heron Island reef are not uniform, there is still much to be learnt about the roles that Archaea play on coral reefs.

We also observed an increase in sequences matching T4-like phages in the two coral associated metagenomes. This is consistent with previous observations that the surfaces of corals are often highly enriched in viruses when compared to surrounding seawater (Seymour et al., 2005; Patten et al., 2008). High microbial abundance and activity within the coral holobiont are expected to provide a host-rich environment for viruses relative to open water conditions and it has been shown that this environment is occupied by a diverse range of viruses including Herpesviridae, Phycodnaviridae, phages and archaeal viruses (Vega-Thurber and Correa, 2011).
Assignment of eukaryotic taxonomy in marine metagenomes is typically problematic due to the scarcity of relevant eukaryotic reference genomes. Our data, which revealed closest matches to organisms not expected to inhabit the coral holobiont, including *Brassicales* (grasses) and *Galliformes* (birds) are consistent with this. However, it is notable that significant increases in matches to *Scleractinia* (hard-coral) sequences were observed in our coral-associated samples, which is supportive of our discrimination of sample types into coral and non-coral associated seawater niches.

Despite overlap in the phylogenetic patterns between coral and non-coral seawater samples, it is notable that the composition of the microbial communities associated with the two coral species also exhibited some differences to each other. This is in accordance with previous work that has shown that different coral species harbor different microbial communities (Rohwer et al., 2001, 2002; Frias-Lopez et al., 2002; Littman et al., 2009). Furthermore, previous work has shown that the microbial communities associated with diseased corals can differ substantially to the communities associated with healthy corals (Bourne et al., 2008, 2011; Sato et al., 2009, 2010; Littman et al., 2011). Although both samples were retrieved from above corals that appeared healthy, without any visible signs of tissue necrosis, colour loss or disease, there remains the possibility that the health status of the two sampled coral niches was different.

Function

The metagenomic data derived from this study provides new insights into the metabolic roles of microbes occupying different niches within a reef. Genes affiliated with core or house-keeping processes were the most abundant metabolic categories across all metagenomes. This is consistent with these general metabolic functions (such as carbohydrate, protein and amino acid metabolism) being essential for microbial survival (Bourne et al., 2005; Venter et al., 2004; Rusch et al., 2007) and is in line with the observations of other metagenomic studied in tropical marine environments (Hewson et al., 2009). Also in-line with these previous studies, we found that substantial differences in less abundant, yet more specialised and dynamic metabolic processes also occurred between the four niches. For example, the two coral seawater metagenomes (lagoon-coral and reef crest-coral) had relatively higher abundances of genes associated with chemotaxis and motility, cell signalling and regulation, and phages, prophages, transposable elements and plasmids, whereas genes associated with photosynthesis were more common in the open water niche. These findings illustrate that microbes inhabiting coral reefs have a general repertoire of core genes, but specific niches promote variability in the importance of more specific functional genes.

Genes relating to phages, prophages, transposable elements and plasmids were most abundant in the reef crest-coral metagenome and were consistently responsible for the greatest significant differences between niches. The increased occurrence of these genes in the reef-crest seawater niche mirrors the increase in sequences affiliated with bacteriophages discussed above and lends support to the importance of viruses within the coral holobiont (Vega-Thurber and Correa, 2011). However, the other coral seawater metagenome, the lagoon-coral niche, had fewer genes associated with this category. This difference between the two coral seawater niches could be attributed to differences in the coral species sampled, differences in the bacterial communities associated with these different coral species, or variability in the health of the corals (Rohwer et al., 2001; 2002; Frias-Lopez et al., 2002; Bourne et al., 2005; Littman et al., 2009).

Genes associated with bacterial motility and chemotaxis also differed significantly between metagenomes, primarily due to their elevated representation in the reef crest-coral niche. While oligotrophic bacteria like SAR 11 and *Prochloroccus* are not motile, many other marine bacterioplankton are highly motile (Mitchell et al., 1995, 1996; Grossart et al., 2001) and the copiotrophic bacteria observed in elevated abundance in the reef crest-coral and lagoon-coral niches, including *Pseudomonas, Vibrio, Shewanella, Pseudoalteromonas* and *Alteromonas* are all motile. It is likely that motility and chemotaxis are particularly important for microbial communities living close to biotic surfaces on coral reefs, where strong chemical gradients are associated with benthic organisms. The chemical products released from corals and algal exudates are often strong chemoattractants for motile marine bacteria (Chet and Mitchell. 1976; Bartlett and Matsumura, 1986; Grossart et al., 2001; Garren et al., 2014). Coral mucus and the exudates of *Symbiodinium* are rich in several organic compounds including amino acids, sugars and dimethylsulfoniopropionate (DMSP) (Von Holt and Von Holt, 1968; Meikle et al., 1988; Raina et al., 2009, 2010; Wild et al., 2005, 2010), which are known chemoattractants for marine bacteria (Miller et al., 2004; Sharp et al., 2012; Stocker and Seymour, 2012), and have recently been shown to attract key coral pathogens (Garren et al., 2014). Microscale gradients in these compounds in the microenvironment immediately adjacent to the coral surface may

promote chemotactic migration of bacterioplankton cells to the coral holobiont (Garren et al., 2014).

Genes associated with regulation and cell signalling were also significantly more abundant in the reef crest-coral metagenome. Not only does coral mucus provide a nutrient-rich environment for microbes within otherwise oligotrophic surroundings, but it can also comprise chemical signals involved in the microbial communities' behaviour and function (Alagely et al., 2011). The high abundance of regulation and cell signalling genes in the coral seawater niches suggests that the microbial community associated with corals engage in higher levels of cell-cell communication than occurs in open water niches. Regulation and cell signalling are likely to be important functions on coral reefs, where bacteria may use signalling processes, including quorum sensing, to organise cellular functions to colonise host organisms including corals and sponges (Taylor et al., 2004; Tait et al., 2010; Alagely et al., 2011; Hunt et al., 2012). Chemical signalling also potentially allows bacteria to defend the holobiont from invading pathogens by altering behaviours such as swarming, biofilm formation and the production of anti-microbial compounds (Rice et al., 2005; Ritche et al., 2006; Shnit-Orland and Kushmaro, 2009; Tait et al., 2010; Mao-Jones et al., 2010; Alagely et al., 2011; Hunt et al., 2012). On the other hand, quorum sensing can regulate virulence in some bacteria (Shnit-Orland and Kushmaro, 2009), which could enable pathogens to more readily infect the host and outcompete beneficial microbes (Rice et al., 2005; Hunt et al., 2012). The elevated occurrence of regulation and cell signalling genes in the coral seawater niches indicate that these processes, which will affect the interactions between microbes and the coral host, are particularly important in the coral holobiont.

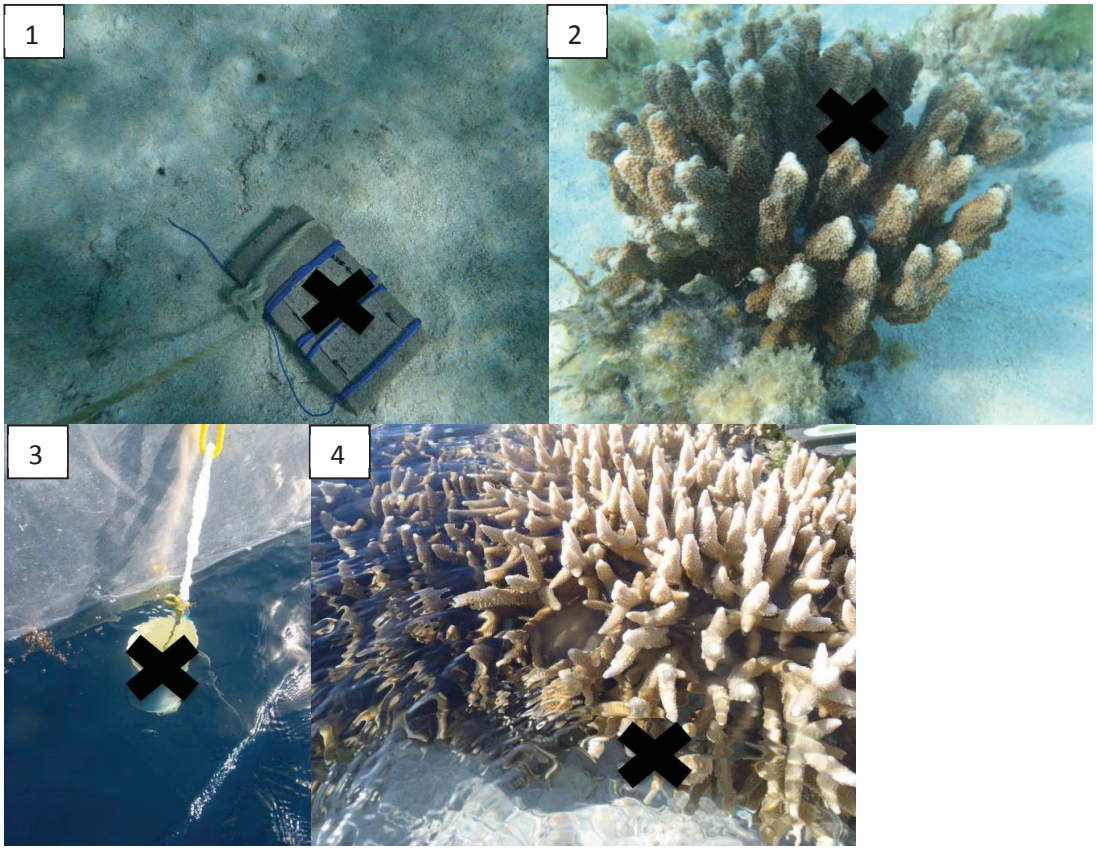
Genes involved in photosynthesis were most abundant in the open water niche with a high proportion of photosynthetic microbes from the genera *Synechococcus* and *Prochlorococcus* underpinning this pattern. Consistent with our findings, Dinsdale et al. (2008) observed shifts in the genes associated with photosystem I and II (PSI and PSII) between coral reef ecosystems. A higher abundance of genes associated with PS I were observed in the open water niche, while PSII associated genes were found to be higher in the reef crest-coral niche. These differences likely reflect subtle

changes in the structure of the photoptrophic microbial community (e.g. shifts in relative importance of *Synechococcus* and *Prochlorococcus*) across the reef.

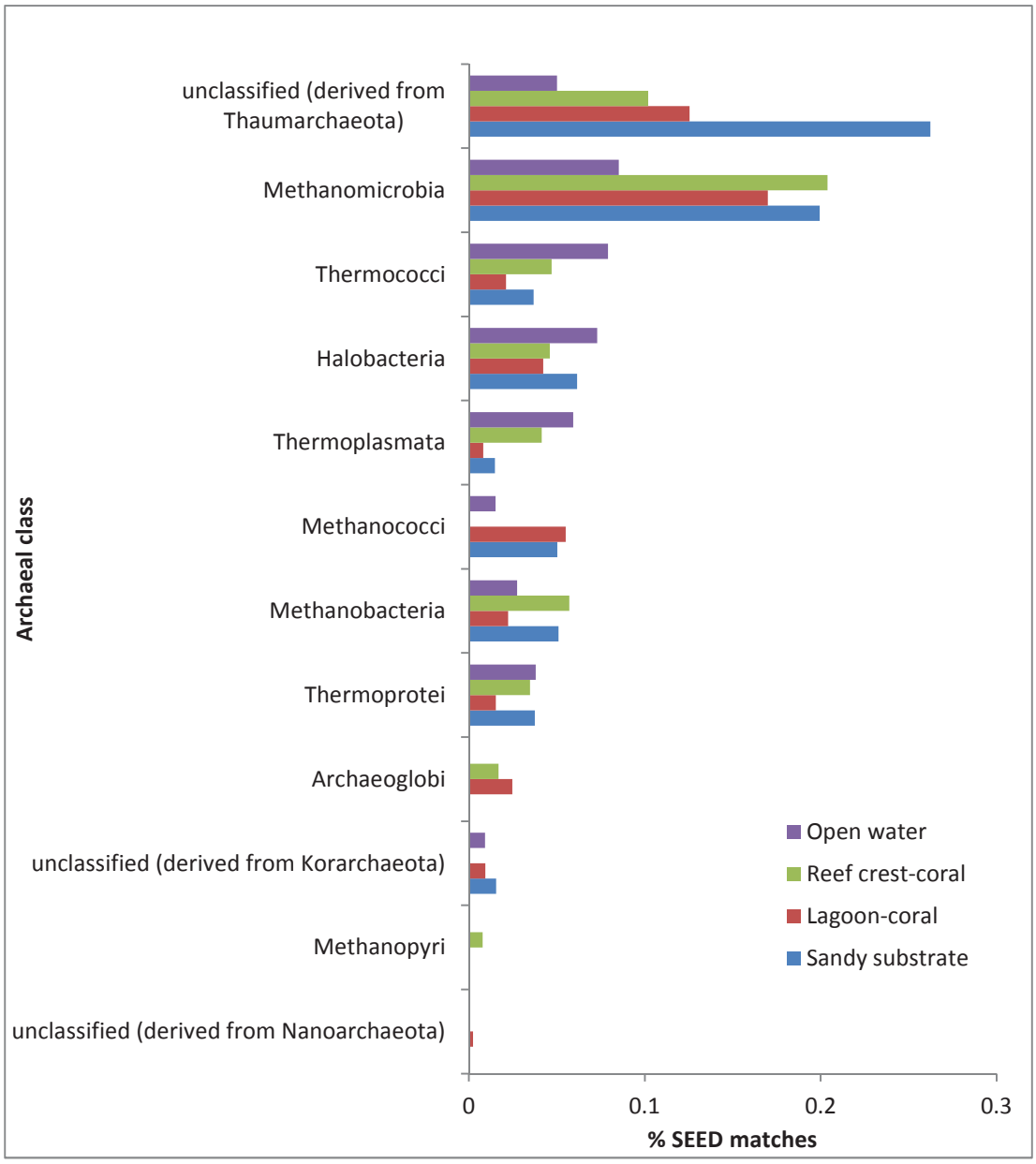
Conclusion

Microbial communities of dissimilar composition and metabolic function were found to occupy different niches within a single coral reef ecosystem. While there were some shared traits across all metagenomes, each niche was characterised by a specific microbial community likely shaped by different conditions, reef structure and occurrence of benthic host organisms. Specifically, the taxonomic shift from oligotrophic to copiotrophic bacteria from open water niches to coral seawater niches within a reef, supports the assumption that benthic reef inhabitants such as corals provide a niche enriched in organic material. Similarly, an enrichment of genes associated with chemotaxis and motility, as well as regulation and cell signalling in coral seawater niches indicates the importance of fine scale chemical gradients emanating from the surfaces of corals in structuring the microbial community. This research has revealed the high level of heterogeneity in species composition and functional capacity of microbial assemblages across a single coral reef ecosystem, highlighting the heterogeneous nature of microbial communities within coral reefs.

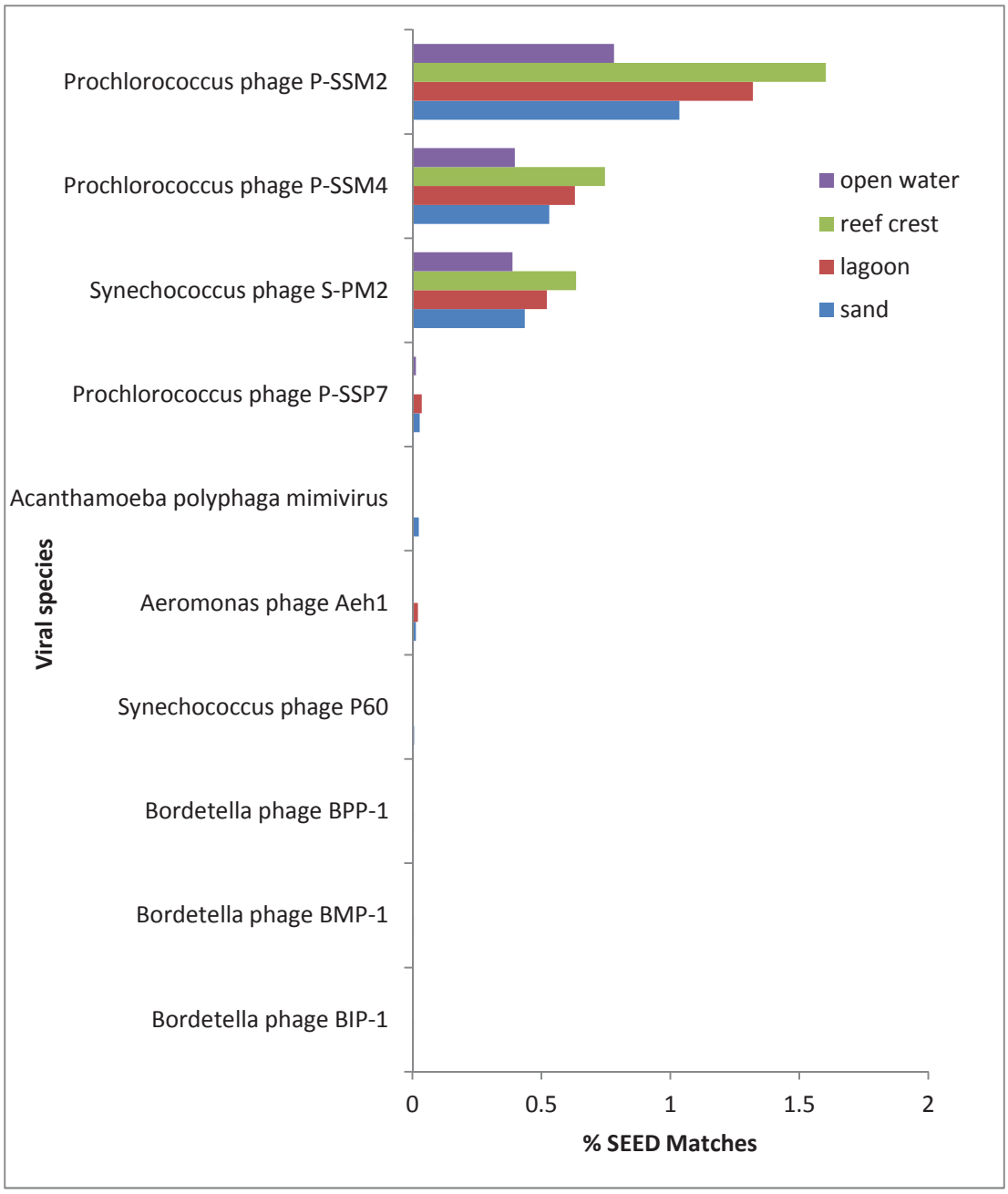
Supplementary Information



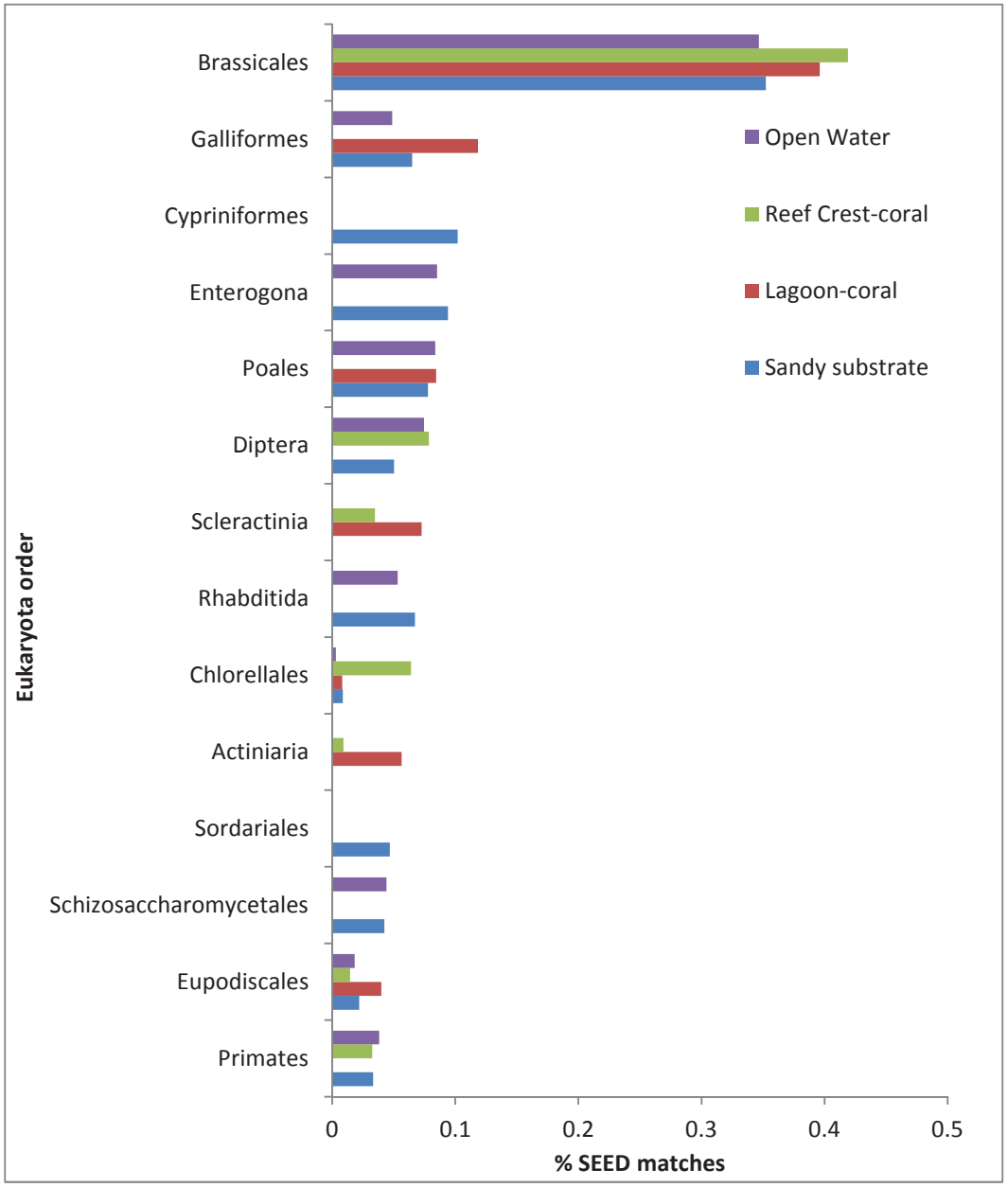
Supplementary Figure S1.1 starting from top left, "x" marks the area from which water was collected from. 1) The sandy substrate niche – following water collection in this niche, the brick was placed above where water was collected for a separate experiment, 2) the lagoon-coral niche: *Acropora palifera*, 3) the open water niche, water was collected prior to where this cylinder was placed for a separate experiment, and 4) the reef crest-coral niche: *Acropora aspera.*



Supplementary Figure S1.2 Relative % of SEED matches to Archaea in each seawater niche metagenome: sandy substrate, lagoon-coral, reef crest-coral and open water (ordered according to frequency of occurrence in each niche)



Supplementary Figure S1.3 Relative % of SEED matches to Viruses in each seawater niche metagenome: sandy substrate, lagoon-coral, reef crest-coral and open water (ordered according to frequency of occurrence in each niche)



Supplementary Figure S1.4 Relative % of SEED matches to Eukaryota greater than 0.05% in each seawater niche metagenome: sandy substrate, lagoon-coral, reef crest-coral and open water (ordered according to frequency of occurrence in each niche)

Supplementary Table S1.1 PRIMER table showing SIMPER analysis of the top seven Genera between coral (lagoon-coral and reef crest-coral) and non-coral (open water and sandy substrate) metagenomes with an average dissimilarity of 9.73. Oligotrophic genera such as *Synechococcus* and *Prochlorococcus* were discriminating genera of the non-coral metagenomes as they had a higher average abundance compared to the coral metagenomes and overall contributed to a cumulative 5.7% of dissimilarity between the metagenomes. Copiotrophic genera such as *Alteromonas, Mycobacterium* and *Vibrio* were shown to be discriminating genera in the coral metagenomes as they had a higher average abundance compared to the non-coral metagenome and contributed up to 3.6% of the dissimilarity between metagenomes.

Groups	**Coral (reef crest & lagoon)**	**Non coral (sandy substrate & open water)**				
Genera	**Average abundance**	**Average abundance**	**Average dissimilarity**	**Dissimilarity/ SD**	**Contribution %**	**Cumulative %**
Synechococcus	1.87	2.76	0.32	1.34	3.33	3.33
Prochlorococcus	1.49	2.12	0.23	1.42	2.32	5.65
Alteromonas	0.88	0.46	0.15	0.95	1.58	7.23
T4-like phages	1.65	1.34	0.11	2.38	1.17	8.40
Ciona (sea squirt)	0.00	0.30	0.11	125.97	1.10	9.50
Mycobacterium	0.75	0.53	0.10	1.41	1.02	10.52
Vibrio	1.51	1.24	0.10	1.27	0.99	11.52

Supplementary Table S1.2: STAMP output represented as a table rather than the STAMP graph due to the large size where Fisher's exact test was used to statistically test for significant differences in the relative representation of microbial genera between the lagoon-coral community and open water community

Genus	Lagoon: rel. freq. (%)	Open Water: rel. freq. (%)	p-values	p-values (corrected)	Effect size	95.0% lower CI	95.0% upper CI
Candidatus Pelagibacter	15.57979677	15.6153636	0.871464117	1.054804928	-0.035566825	-0.453700472	0.381286198
unassigned	4.515839809	4.266364807	0.039476773	0.141033956	0.249475001	0.013285614	0.484056366
Synechococcus	4.49790795	10.2658183 4	6.07700744385e-322	2.692114297 62e-319	-5.767910388	-6.07530761	-5.462947434
Prochlorococcus	2.677824268	6.017018619	2.49E-178	5.52E-176	-3.339194351	-3.580198209	-3.101084844
Pseudomonas	2.546323969	2.041453609	1.02E-08	1.61E-07	0.50487036	0.33309497	0.675298799
Vibrio	1.984459056	1.274444748	1.69E-21	1.50E-19	0.710014308	0.565908441	0.853233455
T4-like viruses	1.908248655	1.268589719	3.51E-18	1.73E-16	0.639658937	0.497138794	0.781221445
Burkholderia	1.872384937	1.459854015	4.61E-08	6.38E-07	0.412530923	0.265693999	0.558077457
Ruegeria	1.624327555	1.383738631	0.000849393	0.004703512	0.240588924	0.100410986	0.379292128
Roseobacter	1.564554692	1.372028573	0.006977063	0.03030234	0.19252612	0.053823038	0.329693676
Shewanella	1.474895397	1.159295835	2.87E-06	2.89E-05	0.315599562	0.184665122	0.445227364
Rhodopseudomonas	1.334429169	1.338850072	0.9592304	1.106612155	-0.004420903	-0.137623338	0.126962934
Brucella	1.150627615	1.208087747	0.368898211	0.661627156	-0.057460132	-0.182989144	0.066149438
Roseovarius	1.025104603	1.032436863	0.907372455	1.071909327	-0.007332261	-0.124551513	0.108048782
Magnetospirillum	1.017632995	1.048050275	0.621323774	0.876580993	-0.030417281	-0.147971549	0.085251701
Rhodospirillum	1.002689779	0.831414185	0.002432922	0.012388327	0.171275594	0.061379413	0.279749038
Rhodobacter	0.948894202	0.96217651	0.832671057	1.033258482	-0.013282308	-0.12637419	0.097954167
Bradyrhizobium	0.907053198	0.927046333	0.734671944	0.971521407	-0.019993135	-0.130869791	0.089009758

Supplementary Table S1.3: STAMP output represented as a table rather than the STAMP graph due to the large size where Fisher's exact test was used to statistically test for significant differences in the relative representation of microbial genera between the lagoon-coral community and the sandy substrate water community

Genus	Lagoon: rel. freq. (%)	Sandy substrate: rel. freq. (%)	p-values	p-values (corrected)	Effect size	95.0% lower CI	95.0% upper CI
Candidatus Pelagibacter	15.57973677	15.68476856	0.55949	0.928292921	-0.104971787	-0.455029568	0.246645487
unassigned	4.515839809	4.680328944	0.112766	0.399642381	-0.164489135	-0.365517192	0.038560324
Synechococcus	4.49790795	5.145832943	9.72E-10	2.05E-08	-0.647924993	-0.852484472	-0.441455136
Prochlorococcus	2.677824268	3.090871626	6.20E-07	7.84E-06	-0.413047358	-0.57255413	-0.251583046
Pseudomonas	2.546323969	2.483000206	0.412302	0.826469523	0.063323763	-0.087159171	0.215991421
Vibrio	1.984459056	1.955678774	0.683029	1.008605458	0.028780281	-0.104596171	0.164353667
T4-like viruses	1.908248655	1.590399946	8.47E-07	1.04E-05	0.317854709	0.191025035	0.447113326
Burkholderia	1.872384937	1.688739861	0.004906	0.031499543	0.183645076	0.056200681	0.313413311
Ruegeria	1.624327555	1.75617706	0.039732	0.179603351	-0.131849505	-0.254905414	-0.006710401
Roseobacter	1.564554692	1.511717213	0.388119	0.803443323	0.052837479	-0.065276153	0.173195326
Shewanella	1.474895397	1.410561415	0.272076	0.677131882	0.064333983	-0.050514121	0.18107216
Rhodopseudomonas	1.334429169	1.271940605	0.266318	0.670333786	0.062488664	-0.04635593	0.173607905
Brucella	1.150627615	1.16516494	0.800045	1.061137787	-0.014537325	-0.116844435	0.089966091
Roseovarius	1.025104603	1.01436787	0.825165	1.071987934	0.010736733	-0.085393413	0.109102428
Magnetospirillum	1.017632995	1.048086469	0.559081	0.931101712	-0.030453475	-0.127007053	0.068277568
Rhodospirillum	1.002689779	0.884176611	0.012712	0.067849538	0.118513168	0.025526339	0.21390296
Rhodobacter	0.948894202	0.948803926	1	1.171957672	9.03E-05	-0.092591014	0.094995344
Bradyrhizobium	0.907053198	0.818612667	0.05352	0.227973071	0.088440331	-0.000378711	0.179634627
Parvibaculum	0.850268978	0.916958582	0.15663	0.492105717	-0.066689604	-0.155792368	0.024529381
Polaribacter	0.809922295	0.791450462	0.677853	1.014489404	0.018471834	-0.066751794	0.105959327
Gramella	0.793484758	1.049023097	6.93E-08	1.02E-06	-0.255538339	-0.345597349	-0.163668664

Supplementary Table S 1.4 PRIMER table showing SIMPER analysis of metabolic categories between coral (lagoon-coral and reef crest-coral) and non-coral (open water and sandy substrate) metagenomes with an average dissimilarity of 1.55. Less abundant metabolic functions including genes associated with Phage function and motility and chemotaxis were found to be discriminating functions of the coral niches (lagoon-coral and reef crest-coral) and overall contributed to a cumulative of 17.7% of the dissimilarity between metagenomes.

Groups	Coral (reef crest-coral & lagoon-coral)	Non-coral (sandy substrate & open water)				
Metabolic Category	Average abundance	Average abundance	Average dissimilarity	Dissimilarity/SD	Contribution %	Cumulative %
Phages, Prophages, Transposable elements, Plasmids	1.43	1.34	0.16	1.49	10.38	10.38
Nucleosides and Nucleotides	1.79	1.69	0.11	1.04	7.40	17.78
Motility and Chemotaxis	0.78	0.72	0.11	1.65	7.32	25.10
Virulence, Disease and Defence	1.25	1.22	0.08	2.31	5.45	30.55
Regulation and Cell signalling	1.10	1.03	0.08	2.24	5.14	35.69
Amino Acids and Derivatives	3.21	3.28	0.07	1.19	4.80	40.49
Carbohydrates	3.54	3.58	0.07	1.55	4.72	45.21
Respiration	1.65	1.59	0.07	0.88	4.40	49.60
RNA Metabolism	2.35	2.40	0.06	1.28	3.61	53.21
Iron acquisition and metabolism	0.88	0.83	0.06	1.40	3.59	56.79
Dormancy and Sporulation	0.32	0.37	0.05	1.69	3.14	59.94
Potassium metabolism	0.49	0.44	0.05	1.27	3.09	63.02
Fatty Acids, Lipids, and Isoprenoids	1.91	1.95	0.04	1.52	2.85	65.87
Stress Response	1.57	1.53	0.04	1.19	2.84	68.72
Miscellaneous	2.80	2.84	0.04	1.47	2.81	71.53
Cofactors, Vitamins, Prosthetic Groups, Pigments	2.55	2.59	0.04	1.97	2.81	74.33
Membrane Transport	1.51	1.47	0.04	1.09	2.79	77.12
Phosphorus Metabolism	1.01	0.99	0.04	1.41	2.65	79.78
Sulphur Metabolism	0.90	0.86	0.04	2.78	2.62	82.40
Metabolism of Aromatic Compounds	0.97	0.93	0.04	1.78	2.46	84.86
DNA Metabolism	1.90	1.89	0.04	1.83	2.44	87.30
Protein Metabolism	2.84	2.88	0.04	0.93	2.44	89.74
Cell Division and Cell Cycle	1.17	1.15	0.03	1.46	2.10	91.84

Chapter 2:

Chemotaxis by Natural Populations of Coral Reef Bacteria

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Abstract

Corals experience intimate associations with distinct populations of marine microorganisms, but the microbial behaviours underpinning these relationships are poorly understood. There is evidence that chemotaxis is pivotal to the infection process of corals by pathogenic bacteria, but this evidence is limited to experiments using cultured isolates under laboratory conditions. We measured the chemotactic capabilities of natural populations of coral-associated bacteria towards chemicals released by corals and their symbionts, including amino acids, carbohydrates, ammonium and dimethylsulfoniopropionate (DMSP). Laboratory experiments, using a modified capillary assay, and in situ measurements, using a novel microfabricated in situ chemotaxis assay, were employed to quantify the chemotactic responses of natural microbial assemblages on the Great Barrier Reef. Both approaches showed that bacteria associated with the surface of the coral species *Pocillopora damicornis* and *Acropora aspera* exhibited significant levels of chemotaxis, particularly towards DMSP and amino acids, and that these levels of chemotaxis were significantly higher than that of bacteria inhabiting nearby, non-coral-associated waters. This pattern was supported by a significantly higher abundance of chemotaxis and motility genes in metagenomes within coral-associated water types. The phylogenetic composition of the coral-associated chemotactic microorganisms, determined using 16S rRNA amplicon pyrosequencing, differed from the community in the seawater surrounding the coral and comprised known coral associates, including potentially pathogenic *Vibrio* species. These findings indicate that motility and chemotaxis are prevalent phenotypes among coral-associated bacteria, and we propose that chemotaxis has an important role in the establishment and maintenance of specific coral–microbe associations, which may ultimately influence the health and stability of the coral holobiont.

Introduction

Corals host bacterial communities that are phylogenetically distinct, more active and more abundant than the bacterial communities in the surrounding seawater (Ducklow and Mitchell, 1979a; Paul et al., 1986; Rohwer et al., 2001, 2002; Frias-Lopez et al., 2002; Kellogg, 2004; Rosenberg et al., 2007; Sweet et al., 2011). Although the recent application of molecular techniques has begun to unravel the complex nature of coral–bacteria interactions (Rohwer et al., 2002; Rosenberg et al., 2007; Ceh et al., 2011, 2012), we only have a rudimentary understanding of the ecological mechanisms and bacterial behaviours underpinning these ecological associations. Recent work focussed on coral pathogens has revealed that chemotaxis may be a potentially important phenotype among coral-associated bacteria (Banin et al., 2001; Rosenberg et al., 2007; Meron et al., 2009; Vega Thurber et al., 2009; Garren et al., 2014).

Chemotaxis may be a particularly beneficial phenotype within reefs because the coral surface microenvironment is characterised by strong gradients of chemical cues and organic material (Kuhl et al., 1995; Mass et al., 2010). Coral mucus and the exudates of the symbiotic dinoflagellate *Symbiodinium* sp. are highly enriched in microbial growth substrates including amino acids, carbohydrates and the organic sulphur compound dimethylsulfoniopropionate (DMSP) (Von Holt and Von Holt, 1968; Ducklow and Mitchell, 1979b; Meikle et al., 1988; Wild et al., 2005, 2010; Raina et al., 2009, 2010, 2013; Garren et al., 2014), and gradients of these materials extend from the coral surface into the immediately surrounding seawater (Garren et al., 2014). The capacity to employ chemotaxis to exploit the resource-rich or infochemical-rich coral surface microenvironment may thus provide considerable advantages for reef microorganisms by enabling access to limiting substrates or potential animal hosts.

Marine bacteria exhibit high-performance motility (Mitchell et al., 1995, 1996; Grossart et al., 2001) and chemotaxis (Stocker et al., 2008; Seymour et al., 2009; Stocker and Seymour, 2012), and there is evidence that bacterial chemotaxis may be an ecologically important phenotype within coral reefs (Banin et al., 2001;

Rosenberg et al., 2007; Meron et al., 2009; Vega Thurber et al., 2009; Garren et al., 2014). Some of the earliest work on marine bacterial chemotaxis demonstrated that coral and *Symbiodinium* exudates are potent chemoattractants (Chet and Mitchell, 1976; Bartlett and Matsumura, 1986), and chemotaxis and motility are important phenotypes for the coral pathogens *Vibrio shiloi* and *V. coralliilyticus* to locate, invade and colonise their coral hosts (Banin et al., 2001; Koren and Rosenberg, 2006; Rosenberg et al., 2007; Meron et al., 2009; Kimes et al., 2011). Recently, it has been demonstrated that *V. coralliilyticus* exhibits extremely strong chemotactic responses towards DMSP to locate heat-stressed colonies of its coral host, *Pocillopora damicornis* (Garren et al., 2014).

Although increasing evidence suggests that chemotaxis is an important phenotype among marine bacterial populations (Blackburn et al., 1998; Stocker et al., 2008; Stocker and Seymour, 2012), most research in this area has relied on the use of cultured isolates and experiments performed under laboratory conditions. Yet, the majority of marine bacteria are not amenable to cultivation, excluding potentially important representatives of natural bacterial communities from laboratory experiments (Jannasch and Jones, 1959; Hoppe, 1976; Bianchi and Giuliano, 1996). In addition, it is important to establish the extent to which swimming and chemosensory capabilities of laboratory isolates reflect their natural state, and how isolates respond to chemoattractants in the presence of natural populations. Here, we aim to expand our understanding of coral–microbe interactions by examining chemotaxis among natural populations of coral-associated bacteria using a combination of laboratory-based and in situ experiments.

Materials and methods

This study was conducted on Heron Island in the Capricorn Bunker Group on the southern Great Barrier Reef, Australia (23°26'S, 151°54'E) during two consecutive winter sampling seasons in July 2010 and July 2011.

Laboratory chemotaxis experiments

To quantify the level of chemotaxis demonstrated by natural communities of coral reef bacteria, we performed a set of laboratory-based studies using seawater samples collected from coral-associated and nearby non-coral-associated environments. Sea-water (1 l) was collected from two environments:

(i) by placing the mouth of a sterile 1 l Schott bottle immediately adjacent (<1 cm distance) to the surface of colonies of the coral species *Pocillopora damicornis*, in 1.5 m depth within the reef of Heron Island (23°126'28''S, 151°155'11''E) ('coral associated') (Supplementary Figure S2.1) and (ii) at the surface of deeper (10 m depth) open water, outside of the Heron Island reef overlaying a large patch of sandy substrate, where no corals were present within a radius of 10 m (23°126'04''S, 151°155'20''E) ('non-coral associated') (Supplementary Figure S2.1). Water samples were returned to the Heron Island Research Station laboratory and used immediately (within 10 min) for chemotaxis experiments.

In these laboratory experiments, we examined chemotactic responses using a modified version of the capillary assay (Pfeffer, 1884; Adler, 1973), whereby sterile 1 ml syringes were filled with 150 ml of putative chemoattractant. Syringes were inserted into 100 ml vials containing seawater collected from the environment (Dennis et al., 2013). Each of three replicates involved a single syringe being inserted into one vial of seawater. Once placed into the vial of seawater chemo-tattractants diffused out of the syringe into the seawater suspension, and chemotactic bacteria within the seawater migrated into the syringe. Putative chemoattractants were selected according to their relevance as components of coral mucus (Von Holt and Von Holt, 1968; Muscatine and Cernichiari, 1969; Ducklow and Mitchell, 1979b; Meikle et al., 1988; Hill et al., 1995; Fitzgerald and Szmant, 1997; Broadbent et al., 2002; Broadbent and Jones, 2004; Wild et al., 2005, 2010; Raina et al., 2009, 2010) and included a suite of amino acids, carbohydrates, dimethylsulfonipropionate and filtered seawater (FSW) (Supplementary Information).

In situ chemotaxis assay (ISCA) experiments

Although the laboratory experiments were designed to provide a first glimpse into the chemotactic capacity of natural communities of coral-associated bacteria, laboratory-based measurements may be influenced by bottle effects, changes in community composition or the change in physical conditions from ocean to the laboratory. To examine coral– microbe chemotaxis within the natural coral reef environment, we complemented the laboratory experiments with in situ chemotaxis measurements, using a newly developed microfluidic-based platform, the ISCA. The ISCA was engineered using soft lithography techniques (Whitesides et al., 2001; Seymour et al., 2008) to create a high-throughput method for chemotaxis quantification in situ, allowing for the simultaneous and replicated testing of multiple chemoattractants under identical conditions, as well as on-chip controls. Each ISCA consists of a matrix of 24 cylindrical wells embedded within a ~30 cm^2 slab of the soft, inert polymer polydimethylsiloxane. Each well has a diameter of 10 mm and a height of 1 mm, resulting in a volume of ~80 ml. Each well has two 1 mm diameter, 5 mm high ports, which are openings connecting the interior of the chamber to the external environment. Individual wells are filled with 80 ml of putative chemoattractant using a pipette. Over the course of a 30 min deployment, the chemoattractant gradually leaks into the external environment through the two inlet ports via molecular diffusion, creating a gradient in the surrounding seawater that triggers the migration of chemotactic bacteria into the wells.

ISCA deployments were conducted 1 year subsequent to the laboratory experiments. The short branches of *P. damicornis* (used in the laboratory experiments) prevented the placement of ISCAs in between the coral branches, meaning that non-intrusive ISCA experiments close to the surface of this coral species were not possible. Consequently, we focussed this component of the study on the abundant coral species *Acropora aspera* and *A. palifera*, which have a more deeply branching structure that allowed for ISCAs to be placed in between coral branches (Supplementary Figure S2.2). ISCAs were deployed on the surface of *A. aspera*, located on Heron Island reef crest, and on the surface of *A. palifera* within the lagoon (Supplementary Figure S2.1). These coral species represent the dominant species

within these two regions of Heron Island reef (Wild et al., 2004). For each coral deployment, ISCAs were carefully inserted into the coral branches (Supplementary Figure S2.2), so that the wells were facing away from the centre of the coral. ISCAs were also deployed on the sandy substrate within Heron Island lagoon and within open water away from any coral (Supplementary Information; Supplementary Figure S2.1). We now describe the bacterial communities inhabiting each of these environments as 'coral-associated' and 'non-coral-associated' bacteria, respectively. For each water-type tested, two ISCAs were deployed in this fashion, so that samples could be collected for both 16S rRNA amplicon sequencing and flow cytometric analysis, respectively. For each ISCA deployment, four chemoattractants were tested simultaneously and each was replicated four times across the ISCA. These included: (1) an equimolar mix (100 mM each) of the amino acids arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Sigma-Aldrich, Sydney, NSW, Australia); (2) an equimolar mix (100 mM each) of the carbohydrates arabinose, fucose, galactose, glucose and mannose (Sigma-Aldrich); (3) 100 mM of ammonium chloride (Sigma-Aldrich); (4) 100 mM DMSP (Tokyo Chemical Industry, Tokyo, Japan). To avoid the generation of secondary chemical gradients, all chemoattractants for both syringe assays and ISCA deployments were diluted in 0.2 mm FSW, using seawater collected from the relevant sampling environment. This FSW was also used as a control in each experiment.

Chemotaxis sample preparation and analysis

For both the laboratory and in situ experiments, the intensity of chemotaxis was determined using flow cytometry to quantify the number of cells that migrated into syringes (laboratory experiments) and ISCA wells (in situ experiments). Upon completion of the assays, samples were immediately (within 10 min) fixed with glutaraldehyde (1% final concentration) for 20 min and frozen in liquid nitrogen before being stored at -80 °C. Samples were stained with SYBR Green I (1:10 000) (Invitrogen, Molecular Probes, Eugene, OR, USA) and analysed using a Becton Dickinson LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Bacterial

populations were discriminated according to SYBR Green fluorescence and side-scatter (Marie et al., 1997; Seymour et al., 2007).

DNA extraction, 16S rRNA gene sequencing and analysis

DNA samples were quick-frozen in liquid nitrogen and stored at - 80 °C. Prior to extraction, DNA samples were quick-thawed in a bath of hot water and a 5 ml aliquot from each sample was transferred into individual microfuge tubes. Genomic DNA was extracted from the samples using MicroLYSIS reagent (Microzone, Haywards Heath, UK) in a 1:5 dilution. The lysis protocol involved one cycle of the following 7-step thermocycling conditions: 65 °C for 15 min, 96 °C for 2 min, 65 °C for 4 min, 96 °C for 1 min, 65 °C for 1 min, 96 °C for 30 s and hold at 20 °C.

The four replicate samples of each chemoattractant across the ISCA designated for DNA collection were pooled to account for biological variability. The composition of the microbial communities responding to each chemoattractant was determined using 16S rRNA amplicon pyrosequencing. Extracted DNA was amplified using universal 16S primers 803F (5'-ATTAGATACCCTGGTAGTC-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') (Supplementary Information). Amplicons were sequenced using the 454 GS-FLX pyrosequencing platform (Roche, Brandford, CT, USA). Homopolymer errors were removed using Acacia (Bragg et al., 2012), and 16S rRNA gene sequences were analysed using the QIIME pipeline (Caporaso et al., 2010; Kuczynski et al., 2011) (Supplementary Information).

Metagenomic analysis of bacterial communities

For each of the four water types where ISCAs were deployed, metagenomes from bulk seawater were also sequenced. For each water type, 10 l of seawater was collected in sterile 10 l Schott bottles (Supplementary Information). Shotgun metagenomic libraries were generated for each of the four samples using the 454 pyrosequencing platform (454 GS-FLX, Roche). Further details of metagenomic sequencing, data quality control and analysis are provided in Tout et al. (2014). For this study, post quality control sequence analysis focussed on genes associated with

bacterial chemotaxis and motility, which were identified by comparing sequences to the KEGG database at the hierarchal functional level one (Supplementary Information).

Chemotactic index and statistical analysis of data

For both laboratory and in situ experiments, the accumulation of bacteria in response to the chemoattractants was expressed in terms of a chemotactic index, I_c (mean±s.d.). The I_c value was calculated by normalising the concentration of cells responding to a given chemoattractant to the number of cells responding to the corresponding FSW control. Positive chemotaxis was identified when I_c was significantly ($P<0.05$) greater than 1. I_c data were tested for normality using the Kolmogorov–Smirnov test, and Levene's test was used to test for homogeneity of variance. In cases where these assumptions were not met, $\log_{10}$ transformations were performed. To compare chemotaxis levels in the laboratory experiments, three-way analysis of variance were used (water type X chemoattractant X concentration). For the ISCA experiments, chemotactic responses within water types were compared using a paired T-test (chemoattractant), whereas a two-way analysis of variance was used to determine differences between the water types (water type X chemoattractant).

A network analysis approach was employed to examine chemotactic preferences at the level of the OTUs (Operational Taxonomic Units). This provided information on whether chemotactic OTUs demonstrated a specialist response, whereby they were only chemotactic to a specific chemoattractant, or a generalist response, whereby they exhibited chemotaxis towards multiple attractants. OTUs responding to the tested chemoattractants were plotted using Cytoscape 2.8.3 (www.cytoscape.org) (Shannon et al., 2003; Smoot et al., 2011; Fuhrman and Steele, 2008; Fan et al., 2012; Needham et al., 2013) according to the QIIME pipeline using the force-directed Cytoscape layout: edge-weighted spring embedded according to e-weights (Kamada and Kawai, 1989; Caporaso et al., 2010; Kuczynski et al., 2011) (Supplementary Information). Known coral-associated OTUs where identified

according to Raina et al. (2010) and were colour coded to highlight their distribution among the chemoattractants.

Differences in the relative abundance of motility and chemotaxis genes between the four metagenomes were identified using Fisher's exact test with a Benjamini false discovery rate multiple test correction, within the statistical analysis of metagenomic profiles package (Parks and Beiko, 2010). All quoted q-values represent corrected values, with only values of <0.05 reported (Parks and Beiko, 2010). Confidence intervals (95%) were determined using the Newcombe–Wilson method. Multivariate statistical software (PRIMER v6) was used to measure the degree of similarity between metagenomes (Clarke and Gorley, 2006). Data were square-root transformed and the Bray–Curtis similarity was calculated between samples. SIMPER analysis (Clarke, 1993) was used to identify the metabolic categories contributing most to the dissimilarity between the metagenomes.

Results

Laboratory chemotaxis experiments

DMSP invoked a strong chemotactic response by bacteria from coral-associated water, as evidenced by cell concentrations in DMSP containing syringes reaching 2–15 times higher values than the control (that is, I_c = 2–15; Figure 2.1a). However, no significant difference was measured in the total number of cells responding to different concentrations of DMSP (Figure 2.1a; Supplementary Table S2.1). Coral-associated bacteria exhibited significantly ($P<0.05$) higher levels of chemotaxis towards all concentrations of DMSP (I_c = 2–15) than the non-coral-associated bacteria, which did not display chemotaxis towards any of the tested DMSP concentrations (I_c = 0.11–0.18) (Figures 2.1a and b; Supplementary Table S2.1).

Among the coral-associated bacteria collected from seawater adjacent to *P. damicornis*, significant ($P<0.001$) positive chemotaxis was also observed towards several of the tested amino acids. The strongest chemotactic response in the coral-associated bacteria was to 1 mM tryptophan (I_c = 28.1±4.9 s.d.) (Figure 2.1c), where

I_c increased with higher concentrations of the attractant and chemotaxis to all concentrations of tryptophan was significantly greater than to the FSW control. Significant (P<0.001) chemotaxis towards all concentrations of aspartic acid (I_c = 5.2±3.4 s.d.) and casamino acid (I_c = 15.0±13.6 s.d.) was also measured (Figure 2.1c; Supplementary Table S2.2), where the 10 mM concentration invoked the highest response in both cases. Responses varied significantly according to concentration, but across all of the tested amino acids there was no general trend of increasing or decreasing I_c with increasing amino-acid concentration. Bacteria from the coral-associated seawater exhibited significantly (P<0.001) higher levels of chemotaxis towards aspartic acid, tryptophan and casamino acids than the non-coral-associated bacteria (Figures 2.1c and d; Supplementary Table S2.2). The non-coral-associated bacteria also showed chemotaxis towards some amino acids, but the levels of chemotaxis were significantly lower (P<0.001). The most potent chemoattractants for these non-coral-associated bacteria also differed (P<0.001) from the coral-associated bacteria and included valine, lysine and threonine (Figure 2.1d). None of the tested carbohydrates induced a significant chemotactic response by coral-associated bacteria (Figure 2.1e; Supplementary Table S2.3), whereas non-coral-associated bacteria showed positive chemotaxis towards all concentrations of arabinose (P < 0.001), with a maximum response (I_c = 5.8±0.7 s.d.) at a concentration of 100 mM (Figure 2.1f; Supplementary Table S2.3).

Table 2.1 Environmental data and metagenome statistics for the four Heron Island microenvironments.

Meta data	Sandy substrate	A. palifera in the lagoon	A. aspera on the reef-crest	Open water
DNA sequences	233,026	201,910	182,182	103,900
Sequences failing QC	23,899	20,691	18,818	10,573
Mean sequence length of DNA base pairs (Post QC)	457	450	449	454
Known annotated proteins	58%	45%	50%	64%
% matches to bacteria	88.4% (58.3% to Proteobacteria)	84% (56.3% to Proteobacteria)	84.6% (60.9% to Proteobacteria)	89.5% (55% to Proteobacteria)
Bulk counts of bacteria per ml, mean +/- SD	9.4×10^4 +/- 1.2×10^4	3.7×10^5 +/- 6.7×10^4	1.0×10^6 +/- 1.6×10^5	3.5×10^6 +/- 4.5×10^5
Seawater temperature	19.7°C	19.7°C	19.6°C	19.7°C

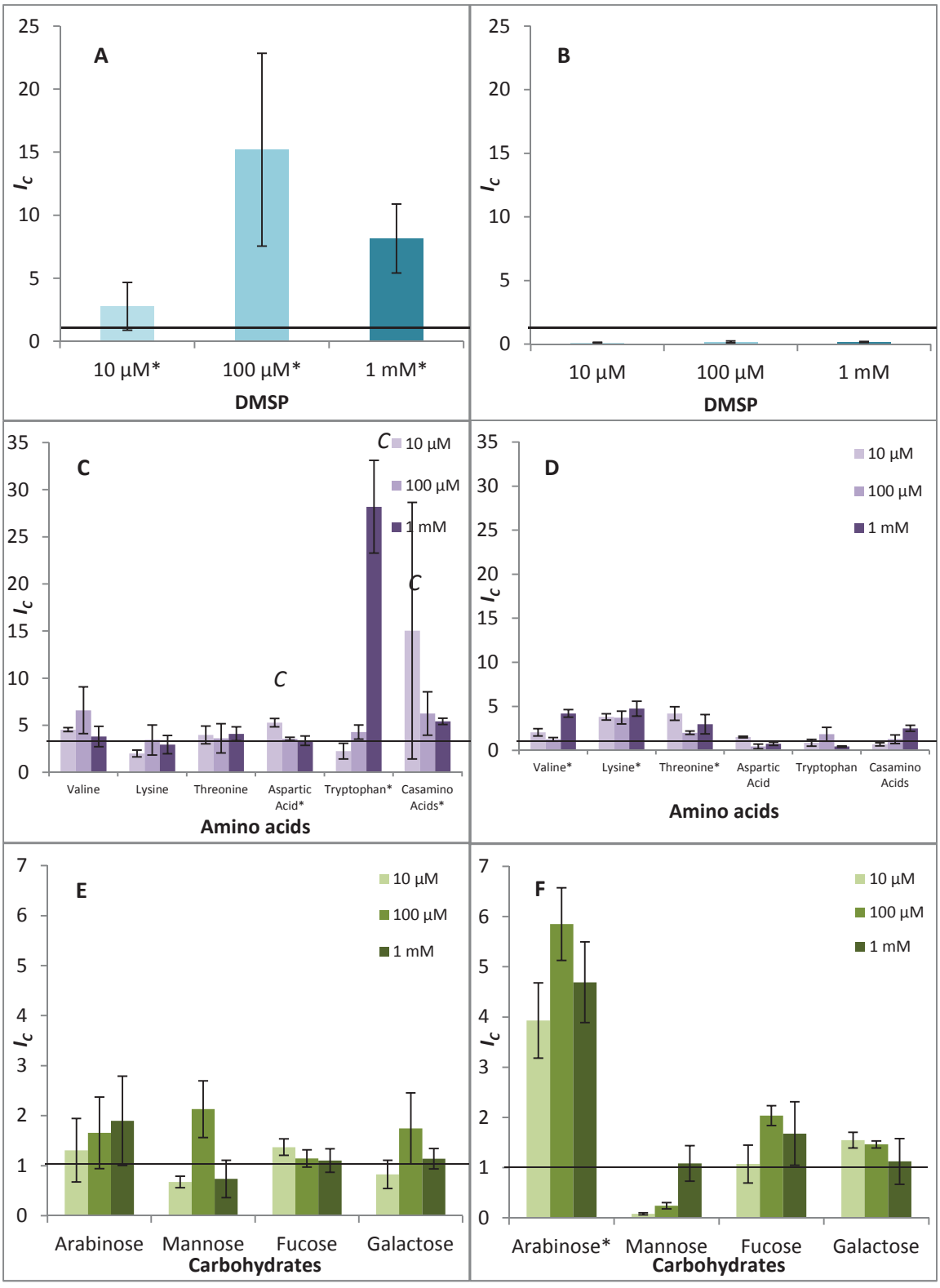


Figure 2.1 Chemotactic index, I_c, of natural bacterial assemblages responding to 10 µM, 100 µM and 1 mM concentrations of DMSP (A, B), amino acids (C, D) and carbohydrates (E, F) and the FSW control (line at 1 on the y axis). Bacterial concentrations in syringes containing chemoattractants were normalised to concentrations in the FSW control. Responses above the horizontal line represent positive chemotaxis. Panels on the left column (A, C, E) represent coral reef-associated bacteria, panels on the right column (B, D, F) represent non-coral-associated bacteria. Vertical bars represent

mean ± SD (n = 3). An asterisk on the x axis represents chemotaxis that is significantly higher than the FSW control; α=0.05. *C* represents the concentration where a significantly highest chemotactic response occurred; α=0.05.

ISCA experiments

For each of the tested chemoattractants, the chemotactic responses were significantly greater in the ISCAs deployed on the surface of *A. aspera* in the reef crest (P<0.001) (Figure 2.2; Supplementary Table S2.4) than at any other water type. For all other tested water types, there was no positive chemotaxis to any chemoattractant (Figure 2.2). However, for the ISCAs deployed on the surface of *A. aspera* in the reef crest, there was significant chemotaxis towards DMSP (I_c = 3.2±0.6 s.d., P<0.05, T = 6.18, Supplementary Table S2.5), the amino-acid mixture (I_c = 25.1±7.9 s.d., P<0.05, T = 7.50, Supplementary Table S6) and ammonium chloride (I_c = 3.5±0.5 s.d., P<0.05, T = 7.97, Supplementary Table S7) (Figure 2.2). There was a response to the carbohydrate mixture (I_c = 8.7±4.3 s.d.), but due to high interreplicate variability this was not significantly different from the FSW control (Supplementary Table S2.8).

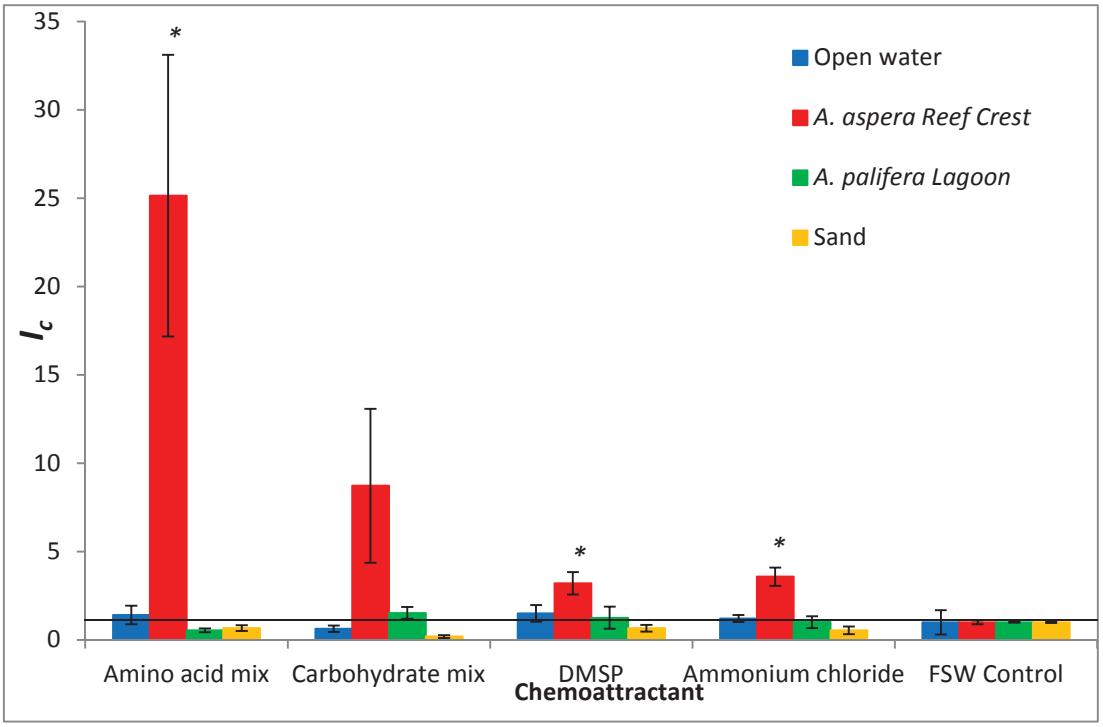


Figure 2.2 Bacterial chemotaxis to amino acid mix, carbohydrate mix, DMSP and ammonium chloride (all at 100 µM) observed at four coral reef microenvironments on Heron Island using the *In Situ* Chemotaxis Assay (ISCA). ISCAs were deployed in the open water outside the reef (blue bars);

on the coral *A. aspera* on the reef crest (red bars); on the coral *A. palifera* in the lagoon (green bars); and at the sandy substrate under water in the lagoon (orange bars) (see Fig. Supplementary S2.1). Values on the y-axis represent the chemotactic index, I_c, computed after bacterial concentrations in ISCA wells containing chemoattractants were normalised to bacterial concentrations in the FSW control. Responses above horizontal line represent positive chemotaxis. Vertical bars represent mean ± SD (n = 4). An asterisk represents chemotaxis that is significantly higher than the FSW control; α=0.05.

Community composition of chemotactic bacteria from the ISCAs

The composition of the chemotactic bacteria in the *A. aspera* ISCA deployments conducted on the reef crest (Figure 2.3) differed from the community composition in the surrounding sea-water (Supplementary Figure S2.3). The background seawater sample was dominated by microorganisms that are typically the most abundant members of bacterial communities within oligotrophic tropical waters, including *Candidatus pelagibacter* (SAR11) and *Synechococcus* (Supplementary Figure S2.3). At the family level, the community retrieved from the ISCA wells were relatively conserved across the chemoattractants tested and was dominated by members of the *Rhodobacteraceae, Comamonadaceae, Flavobacteriaceae, Pseudomonadaceae* and *Sphingomonadaceae* (Figure 2.3; Supplementary Figure S2.4a). However, some less-abundant families displayed a more specialized response, with representatives responding to only one or a subset of the tested chemoattractants (Supplementary Figure S2.4b). For example, *Vibrionaceae* responded only to amino acids, carbohydrates and DMSP, whereas the *Shewanellaceae* responded only to DMSP and carbohydrates (Figure 2.3).

The extent of 'generalism' versus 'specialism' in the chemotactic responses to individual chemoattractants was probed more extensively at the OTU level using network analysis (Figure 2.4). Of the 394 OTUs responding to the tested chemoattractants, only 4.3% responded to all four compounds (the nodes in the centre of the network in Figure 2.4) and can be considered 'generalist chemotaxers'. Of this 'generalist' community, 65% were identified as belonging to known coral-associated families. On the other hand, 19.3% of OTUs responded to two or three chemoattractants, of which 44% were from known coral-associated families. The

bulk of OTUs (76.4%) exhibited a 'specialist' response, being attracted to only one compound, 39.5% of which belonged to known coral-associated families. Of these, 42.5% responded to the carbohydrate mixture only, 24.9% to the amino acid mixture only, 16.6% to DMSP only and 15.9% to ammonium chloride only. These specialist OTUs included a diverse range of organisms often belonging to families that contain known coral associates, including *Endozoicomonaceae, Alteromonadaceae* and *Vibrionaceae* (Figure 2.4).

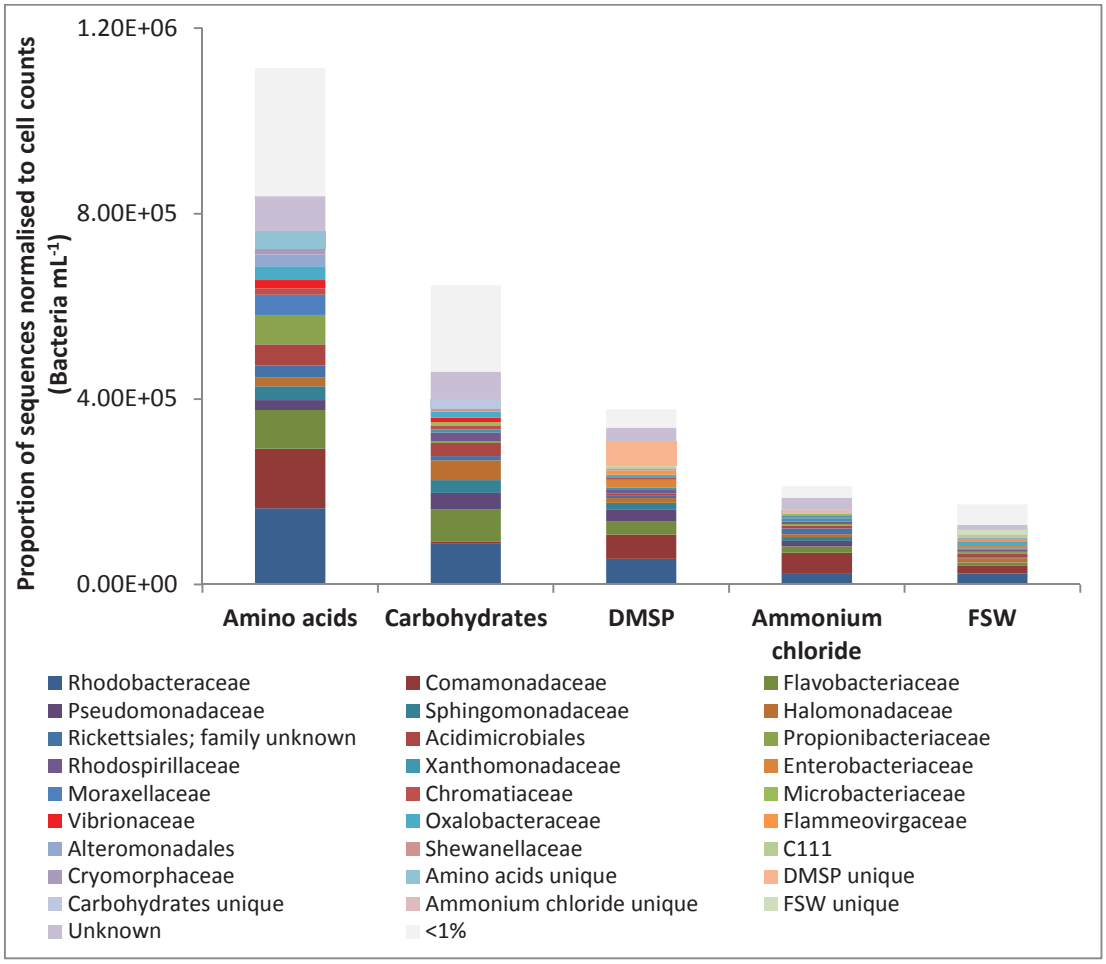


Figure 2.3 Taxonomic identities (family) of chemotactic bacteria responding to the ISCA deployments on the reef-crest coral A. aspera using 16S rRNA gene sequencing, where hits were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME. The relative proportions of families responding to each chemoattractant were normalised by multiplying by the cell abundance retrieved from FCM (Figure 2.1) (Dennis et al., 2013). The microbial community identified in the FSW control is representative of organisms that swam into this treatment as a consequence of random motility, rather than chemotaxis, due to the lack of any chemical gradient. As such, this sample provides an overview of the motile, but not necessarily chemotactic proportion of the community. For more detail on unique taxa responding to individual chemoattractants, see Supplementary Figure S2.3.

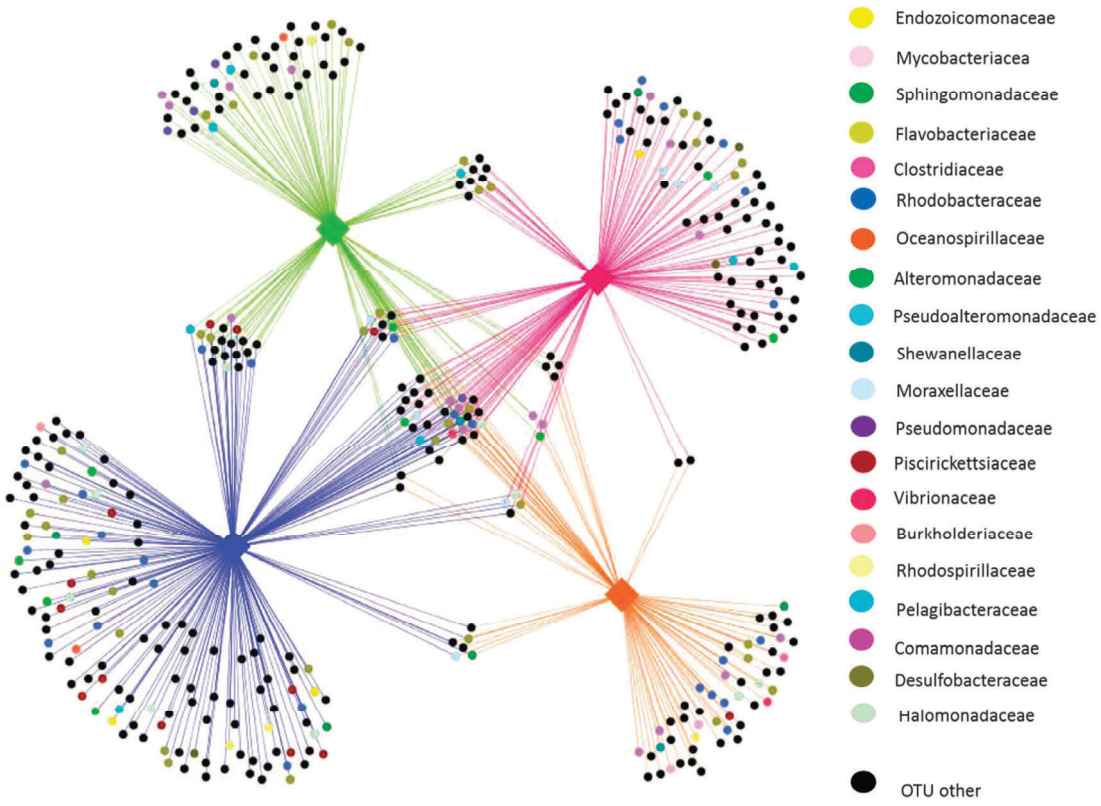


Figure 2.4 OTU network of chemotactic bacteria (black nodes) responding to the chemoattractants (coloured nodes) connected by the same coloured edges used in the ISCA deployment on *A. aspera* on Heron Island's reef crest, Great Barrier Reef. When an OTU responded only to a single chemoattractant its corresponding node is connected only to that chemoattractant (the outer nodes). Where an OTU responded to multiple chemoattractants, the corresponding node is connected to the relevant groups of chemoattractants (nodes in the centre).

Chemotaxis gene patterns in metagenomes

Patterns in chemotaxis and motility genes obtained from metagenomic data closely reflect the phenotypic patterns observed in each of the chemotaxis experiments. Genes encoding cell motility and chemotaxis, including the chemotaxis genes cheA, B, D, R, V, W, Y, Z, the methyl-accepting chemotaxis gene MCP, the motility genes motA, B, C and the flagellar proteins flgA, B, D, E, G, H, I, L, M, flhA, B, C, D and fliC, F, G, I, M, N, Y, O, Z, P, R, were responsible for driving significant differences between the coral-associated and the non-coral-associated metagenomes

(Supplementary Tables S2.9–S2.11). These motility genes were over-represented in the coral-associated water types, comprising 0.83% of the total number of sequences (Table 2.1), compared with only 0.23% in the open-water type (Figure 2.5a). Consistent with the significantly higher levels of chemotaxis observed in *A. aspera* in the reef crest, the above mentioned genes were also generally significantly ($q<0.05$, Fisher's exact test) over-represented on the surface of *A. aspera* (Figures 2.5a and b). In *A. aspera*, MCP, cheA, motA, motB, cheB, cheR and cheY were significantly ($q<0.05$) over-represented relative to the open-water type. The differences between the two coral-associated samples were less pronounced: MCP was the only gene significantly over-represented in *A. aspera* on the reef crest relative to *A. palifera* in the lagoon ($q<0.05$) (Figure 2.5b).

Discussion

Our data provide the first direct in situ measurements of chemotactic behaviour among natural populations of coral-associated bacteria, with high levels of chemotaxis demonstrated towards a range of coral holobiont-related chemicals. These in situ measurements add a new dimension to existing evidence that some coral pathogens are chemotactic towards coral mucus and other host-derived compounds (Banin et al., 2001; Koren and Rosenberg, 2006; Rosenberg et al., 2007; Meron et al., 2009; Kimes et al., 2011; Vidal-Dupiol et al., 2011; Garren et al., 2014), and that chemotaxis and motility genes can be prominent and dynamic features in the metagenomes of coral–microbial communities (Vega Thurber et al., 2009). Given the strong chemotactic responses that we observed in the laboratory experiments and ISCA deployments, we propose that motility and chemotaxis may be important phenotypes within the context of coral– microbial interactions.

Our laboratory based and in situ measurements indicate that coral reef bacteria are well equipped to sense and respond to the chemicals released from corals. The diffusion of chemicals away from the coral surface into the surrounding seawater generates microscale chemical gradients extending into the adjacent seawater, whereas the shedding of carbon-rich mucus by corals may produce chemical hotspots

Wild et al., 2004). Chemotaxis is thus likely to be an important mechanism favouring the colonisation of coral surfaces by bacteria.

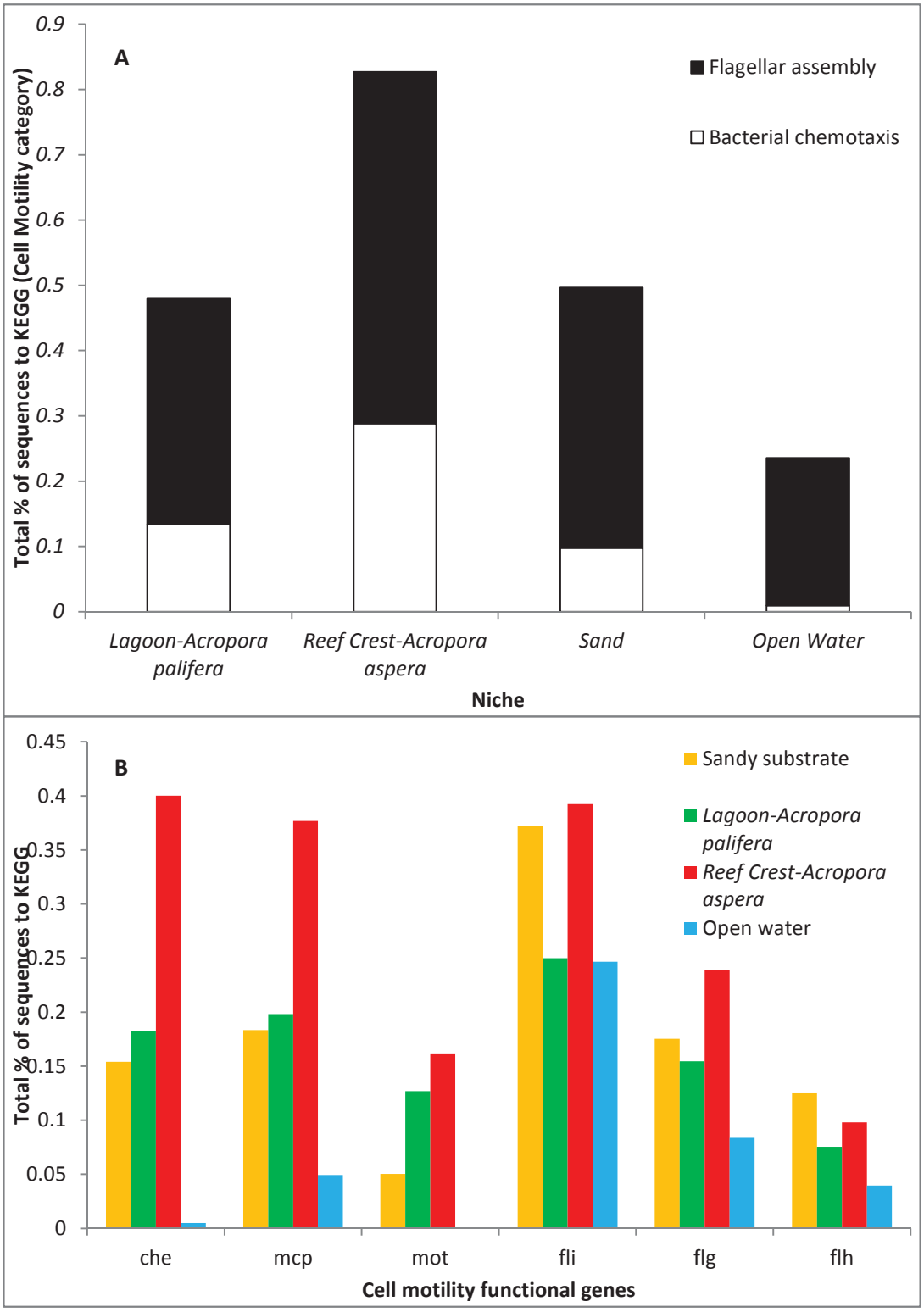


Figure 2.5 Functional categories of motility and chemotaxis composition of four metagenomic libraries derived from different reef habitats on Heron Island by comparing the sequences with BLASTn to the KEGG database; a) cell motility category, and b) functional genes associated with cell

motility category. Relative representation in the metagenome was calculated by dividing the number of hits to each category by the total number of hits to all categories, thus normalizing by sequencing effort.

The laboratory-based syringe assays revealed a much stronger chemotactic response by coral-associated bacteria, obtained from near the surface of the coral species *P. damicornis*, than by bacteria collected from a non-coral site. In addition, the bacteria from the coral- and non-coral-associated sites showed different preferences for chemoattractants, indicating that the chemical requirements, or use of chemical signals as chemotactic cues, vary between coral-associated and nearby open-water bacterial communities. These patterns were supported by the results of the *in-situ* experiments, whereby bacteria associated with the surface of the coral *A. aspera* exhibited significantly higher levels of chemotactic capability than bacteria from any other water type around the reef or from the open water.

The short-branching morphology of *P. damicornis* was not amenable to ISCA deployment, so ISCA experiments were performed on the more deeply *branching A. aspera* in the reef crest and *A. palifera* in the lagoon. Although this prevents direct comparison of our laboratory and *in-situ* results, the general pattern of higher levels of chemotaxis on coral surfaces was conserved between approaches.

Elevated levels of chemotaxis observed among the microbial communities associated with the reef crest coral *A. aspera* in the ISCA experiments were supported by metagenomic analysis, where genes associated with motility and chemotaxis were significantly more abundant in the seawater associated with *A. aspera* than in any other sample. Of note, motility and chemotaxis genes in the *A. aspera* sample were significantly more abundant than in the sample obtained from the other coral species *A. palifera* located in the lagoon, which is directly in line with the differences in chemotactic responses observed between the bacterial communities associated with these two coral species in the ISCA experiments. Taken together, our results demonstrate that chemotaxis is heterogeneous across a coral reef and between communities associated with different corals. The different chemtoaxis patterns

observed between the bacterial communities associated with the two *Acropora* species may be due to inter-coral variability in microbial community composition and function (Rohwer et al., 2001, 2002; Tout et al., 2014), or slight differences in the morphology of the two coral species may have altered the biophysical environment inhabited by the resident microbes (Wallace, 1999). In addition, the location of the corals within the Heron Island reef system may be responsible for some of these differences. *A. aspera* was chosen for sampling in the reef crest environment, whereas *A. palifera* was chosen within the lagoon, because these species dominate the coral communities within these two habitats, respectively (Wild et al., 2004). The reef crest, where the *A. aspera* deployment occurred is an environment characterised by a wall of Acropora corals, where 69% of the benthos is covered by hard corals (Salmond et al., 2013).

In contrast, in the lagoon where the ISCAs were deployed on *A. palifera*, hard corals contribute to only 6% of the total benthic cover (the lowest hard coral cover on Heron Island) (Salmond et al., 2013) and the bulk of the substrate is sand. As a consequence, the local environment surrounding the different coral colonies varied substantially, with differences in the bulk microbial community within these two habitats potentially contributing to the differences observed here. Finally, Vega Thurber et al. (2009) showed significant shifts in chemotaxis genes among the microbial communities associated with stressed corals relative to healthy individuals, and although no notable signs of disease or stress were apparent in either coral tested here, there remains the possibility that differences in the health status of the coral hosts may contribute to differences in the level of chemotaxis observed here.

Natural communities of coral-associated bacteria showed strong chemotactic responses towards DMSP in both the laboratory assays and the ISCA deployments. This is consistent with DMSP being an important source of carbon and reduced sulphur for marine bacteria (Howard et al., 2006) and a chemical cue for some coral pathogens (Garren et al., 2014). DMSP is abundant on coral reefs (Broadbent et al., 2002; Broadbent and Jones, 2004; Raina et al., 2010), with coral mucus concentrations (up to 62 mM) the highest reported in the marine environment (Broadbent et al., 2002; Broadbent and Jones, 2004). There is now evidence that both

the coral symbionts *Symbiodinium* spp. (Keller et al., 1989; Broadbent et al., 2002) and the coral animal (Raina et al., 2013) have the capacity to produce significant quantities of DMSP. Our results confirm that, similar to other marine microorganisms (Miller et al., 2004; Seymour et al., 2010; Sharp et al., 2012), coral-reef-associated bacteria use chemotaxis to enhance their access to DMSP or to follow DMSP gradients as a cue to locate the host (Garren et al., 2014). The dominant bacterial taxa responding to DMSP included known coral-associated species that have the capacity to degrade DMSP (*Flavobacteriaceae*) and DMS (*Comamonadaceae*) or both (*Rhodobacteracea, Pseudomonaceae* and *Halomonaceae*) (Raina et al., 2010). It is notable that we also observed some *Vibrio* sequences in the DMSP sample, because recent evidence suggests that DMSP can be a potent chemoattractant for coral pathogens belonging to this genus (Garren et al., 2014).

Strong chemotactic responses to several amino acids by coral-associated bacteria were also observed using both the syringe assays and the ISCA deployments. The amino acids used in the chemotaxis assays have previously been found in the mucus of several coral species (Ducklow and Mitchell, 1979b; Meikle et al., 1988; Fitzgerald and Szmant, 1997). Chemotaxis towards amino acids is well recognised among enteric bacteria (Adler, 1966; Mesibov and Adler, 1972; Kim et al., 2001; Bainer et al., 2003; Frank et al., 2011), has been demonstrated in some marine bacteria (Barbara and Mitchell, 2003a,b) and our results indicate that it may also be involved in coral–microbe interactions in coral reef environments.

Marine bacteria have been shown to exhibit chemotaxis towards carbohydrates present in the exudates of phytoplankton (Hellebust, 1965; Bell and Mitchell, 1972) and in the mucus of the Hawaiian squid *Euprymna scolopes* light organ (DeLoney-Marino et al., 2003). Despite the prominence of carbohydrates in coral mucus (Ducklow and Mitchell, 1979b; Meikle et al., 1988; Wild et al., 2005), the chemotactic response of coral-associated bacteria towards the tested carbohydrates in both the syringe assays and the ISCAs were never significantly different from the FSW control. Taken together, these findings indicate that carbohydrates previously

shown to occur in coral mucus do not appear to be an important chemical cue for coral-associated bacteria.

Our results demonstrate that natural populations of coral-associated bacteria exhibit chemotaxis towards ammonium. Aquatic heterotrophic bacteria have previously been shown to use chemotaxis to exploit patches of inorganic substrates including ammonium (Stocker and Seymour, 2012; Dennis et al., 2013). Within coral reefs, nitrogen is often a limiting nutrient in the water column (Thomas and Owen, 1971; Crossland et al., 1984; Moore et al., 2013), as nitrate and ammonium concentrations are often <2 mM (Crossland et al., 1984; Bythell, 1990). However, ammonium and other inorganic nutrients are likely to be generated as metabolic by-products in the coral holobiont (Kawaguti, 1953; Muscatine and D'Elia, 1978; Siboni et al., 2008), and ammonium levels can reach up to 50 mM within coral mucus (Wild et al., 2005). Therefore the ability to use chemotaxis to exploit elevated levels of inorganic nutrients near the surface of corals may provide a competitive advantage for some coral reef bacteria.

The microbial community identified in the FSW control is representative of organisms that swam into this treatment as a consequence of random motility, rather than chemotaxis, due to the lack of any chemical gradient. As such, this sample provides an overview of the motile, but not necessarily chemotactic proportion of the community. The choice of chemoattractant concentrations in our experiments was guided by concentrations previously shown to occur within coral microenvironments, such as coral mucus (Broadbent and Jones, 2004; Wild et al., 2004, 2005, 2010). In most cases, no significant difference was observed in the chemotactic response between different concentrations. However, the coral-associated bacteria's response to tryptophan increased with increasing concentrations. On the other hand, in the case of aspartic and casamino acids, the strongest chemotaxis occurred in response to the lowest tested concentration of 10 mM, which is perhaps indicative of an inhibitory response associated with saturation of chemoreceptors at higher concentrations (Mesibov et al., 1973). These patterns indicate that the chemotactic sensitivities and thresholds for coral-associated bacteria vary between different types

of compounds, which may reflect the relative availability and concentrations of these substrates in the environment.

Recent measurements of the chemotactic behaviour of a natural bacterial population from a lake revealed a strong phylogenetic partitioning in the response towards inorganic compounds (Dennis et al., 2013). Our results have expanded upon this work by identifying members of the chemotactic bacterial communities associated with the coral species *A. aspera*, and demonstrating that these differed substantially from the community in the surrounding seawater. This indicates that chemotaxis may act as a behavioural filter, favouring certain species over others in associating with corals and thus determining the composition of microbial communities within specific coral microniches.

At the family level of taxonomic resolution, we found that the same bacterial groups were typically the dominant responders to all chemoattractants, indicating that microorganisms belonging to a restricted range of bacterial families perform chemotaxis in the coral reef environment. These groups included families with members that have metabolic requirements for the tested chemoattractants (for example, *Flavobacteriaceae* for DMSP; Raina et al., 2010) or are known coral associates (*Rhodobacteraceae, Comamonadaceae, Flavobacteriaceae* and *Pseudomonadaceae* (Raina et al., 2010; Morrow et al., 2012)). However, at a finer taxonomic resolution it became clear that specialist chemotactic responses, whereby organisms only exhibited chemotaxis to certain chemicals, were common. There were some families where representatives only responded to one or a subset of the chemoattractants tested. These more-specialised groups of chemotaxers included members of the *Shewanellaceae* and *Vibrionaceae* families. The *Shewanellaceae* only exhibited chemotaxis towards DMSP and carbohydrates. This family includes known coral-associated bacteria (Shnit-Orland and Kushmaro, 2009; Raina et al., 2010; Ceh et al., 2012) and species that degrade DMSP (Raina et al., 2010). Chemotaxis by members of the marine *Shewanellaceae* towards amino acids and algae has previously been observed (Barbara and Mitchell, 2003a,b), and our results demonstrate that members of this known group of coral associates may use chemotaxis towards DMSP within the coral holobiont.

Clear differentiation of the chemotactic community into groups of 'specialist' and 'generalist' chemotaxers became particularly evident when responses were assessed at the OTU level. Approximately 4.3% of all OTUs responded to all four compounds tested, whereas 19.3% responded to 2 and 3 chemoattractants. This suggests that a subset of the chemotactic community is made of 'generalist chemotaxers' that have the capacity to sense and direct movement in response to diverse chemicals compounds. Currently, little is known about the chemoreceptors used by marine bacteria (Stocker and Seymour, 2012), but the generalist chemotaxers identified here may either have multiple sets of chemoreceptors, which allow them to respond to a variety of compounds (Wadhams and Armitage, 2004; Parales et al., 2013), or single chemoreceptors that allow for binding of multiple compounds (Adler, 1969; Glekas et al., 2012). However, the bulk (76.4%) of chemotactic OTUs identified here can be classified as 'specialist chemotaxers', as they responded to only one compound. Many of these specialist chemotaxers were also known coral associates including members of *Endozoicomonaceae, Rhodobacteraceae, Vibrionaceae* and *Mycobacteriacea*. In the environment, the heterogeneity in chemotactic responses observed here could lead to strong niche partitioning among the bacterial community, suggesting that this behaviour could underpin some of the heterogeneity in microbial community composition previously observed between different coral hosts (Rohwer et al., 2002).

The partitioning of coral–microbe communities observed here may be encouraged by differential release of chemoattractants from the coral holobiont under varying environmental conditions. There is evidence that the production and release of key chemoattractants, such as DMSP, can vary markedly under different environmental conditions and coral health states (Raina et al., 2013; Garren et al., 2014). From a bacterium's perspective, chemotaxis serves a variety of potential ecological functions, including (i) providing cells with greater access to important growth substrates and nutrients in otherwise oligotrophic habitats (Stocker et al., 2008; Seymour et al., 2009), (ii) enhancing exposure to terminal electron acceptors/donors (Schweinitzer and Josenhans, 2010) or (iii) use of infochemicals that may provide pathogenic microorganisms with information about the location, health and potential

susceptibility of coral hosts to infection (Garren et al., 2014). Heterogeneity in the strength of chemotaxis towards different chemoattractants observed across the different water types is perhaps indicative of varying ecological strategies among microbial communities on coral reefs.

We have provided the first *in-situ* quantification of bacterial chemotaxis on a coral reef. Previous studies have shown that different habitats and microenviroments on coral reefs host phylogenetically distinct communities (Rohwer et al., 2002; Rohwer and Kelly, 2004), but our results show that different reef features (for example, coral species and water types) also host distinct microbial phenotypic capacities, specifically the ability to perform chemotaxis. In addition, we have provided a direct mechanism for how chemical gradients associated with coral surfaces may be involved in the establishment of specific coral–bacterial relationships, and how microbial chemotaxis might shape the composition of coral reef bacterial communities. As such, we suggest that within the chemically and physically complex coral microenvironment, bacterial behaviours, including motility and chemotaxis, may be fundamental drivers of patterns in microbial diversity and metabolism, coral infection dynamics and chemical cycling processes.

Supplementary Information

Syringe assays

For the syringe assays the following chemoattractants were used the amino acids tryptophan, aspartic acid, casamino acid, lysine, valine, and threonine (Sigma-Aldrich) (Von Holt & Von Holt, 1968; Ducklow & Mitchell, 1979b; Meikle et al., 1988; Fitzgerald & Szmant, 1997), the carbohydrates arabinose, mannose, fucose and galactose (Sigma-Aldrich) (Muscatine & Cernichiari, 1969; Ducklow & Mitchell, 1979b; Meikle et al., 1988; Wild et al., 2005; 2010), and the organic sulphur compound dimethylsulfoniopropionate (DMSP) (Tokyo Chemical Industry) (Hill et al., 1995; Broadbent et al., 2002; Broadbent & Jones, 2004; Raina et al., 2009, 2010, 2013; Garren et al., 2014). All attractants were diluted in 0.2 µm filtered seawater (FSW) to final concentrations of 10 µM, 100 µM and 10 mM. These concentrations were chosen to cover the range of concentrations produced by the coral or zooxanthellae (Hill et al., 1995), or measured on the surface of corals (Hill et al., 1995; Broadbent et al., 2002; Broadbent & Jones 2004; Raina et al., 2009, 2010, 2013).

In situ Chemotaxis Assays (ISCAs)

Two ISCAs were placed into the branches of *Acropora palifera* within the lagoon (23°26'41"S, 151°54'47"E) (Fig. S1) and another two ISCAs were inserted into the branches of *Acropora aspera* colonies located on the reef crest (23°26'41"S, 151°54'47"E) (Fig. S1). These coral species were chosen because they represent the dominant species in the respective microenvironments of the Heron Island reef (Wild et al., 2004). Two further ISCAs were deployed, facing upward, on the sandy substrate within the Heron Island lagoon (23°26'36"S, 151°54'47"E), in a location where the water depth was 40 cm and there were no corals present within a radius of 10 m (Fig. S1). The final set of two ISCAS were deployed in open water to sample surface water communities, ~3 km outside of Heron Island's north-western fore-reef slope (23°24'58"S, 151°53'12"E), where the water depth was 40 m (Fig. S1). For the

open water deployment, ISCAs were held in place by a purpose-designed frame. To minimise advective effects the open-water deployment was carried out under Lagrangian conditions, whereby the vessel and ISCA moved with the bulk seawater flow. Deployment at all locations occurred over a 30 minute period on an incoming tide at the same time of day over the course of four consecutive days, approximating the same time point within the tidal cycle.

DNA Extraction, 16S rRNA Gene Sequencing and Analysis

Extracted DNA was amplified using universal 16S primers 803F (5'-ATTAGATACCCTGGTAGTC-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') under the following cycling conditions: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 90 s; followed by a final extension at 72°C for 10 min (Engelbrektson et al., 2010).

Briefly, DNA sequences were de-multiplexed and reads shorter than 200 bp, with a quality score <25 or containing homopolymers exceeding 6 bp were discarded. Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar et al., 2010) and taxonomy was assigned against the Greengenes database (version 13_5; McDonald et al., 2012) using BLAST (Altschul et al., 1990). OTUs occurring in the microLYSIS control were removed from the dataset and chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011) and filtered from the dataset.

Metagenomic Analysis of Bacterial Communities

For the *A. palifera, A. aspera,* and sandy substrate samples, seawater was collected by placing the mouth of the bottle immediately over (<1 cm distance) the surface of the corals and the substrate. Samples were filtered onto 0.2 µm polycarbonate membrane filters (Millipore) within 10 min of collection. The filters were frozen at -80°C until DNA extraction was conducted using a bead beating and chemical lysis

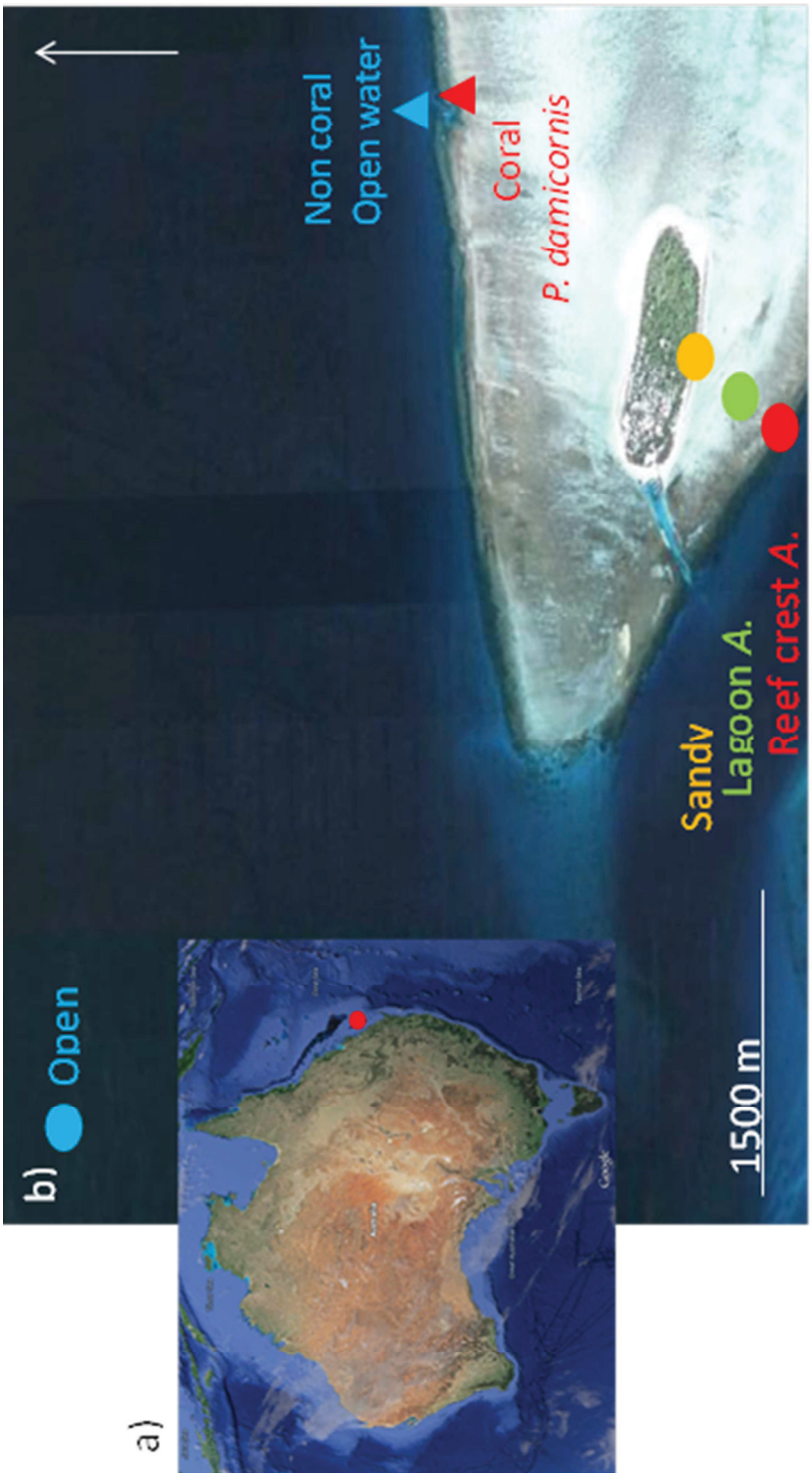
kit (MO BIO PowerWater, MO BIO, Carlsbad, California, USA) according to the manufacturer's instructions.

The taxonomic identity of microorganisms from seawater collected from the reef-crest *A. aspera* microenvironment were identified by comparing sequences to the Greengenes database at the genus level using the *Meta Genome Rapid Annotation using Subsystems Technology* (MGRAST; Version 3) pipeline (Overbeek et al., 2005; Meyer et al., 2008). Matches with an E-value of <0.05 were considered significant with a minimum alignment length of 50 bp (Vega-Thurber et al., 2009; Edwards et al., 2010; Pfister et al., 2010; Poroyko, 2010; Delamont et al., 2011; Jeffries et al., 2011, 2012; Seymour et al., 2012; Smith et al., 2012). Sequences were then rarefied to the same depth (the lowest number of sequences in a sample) to remove the effect of sampling effort upon analysis (Santos et al, 2014).

Chemotactic Index and Statistical Analysis of Data

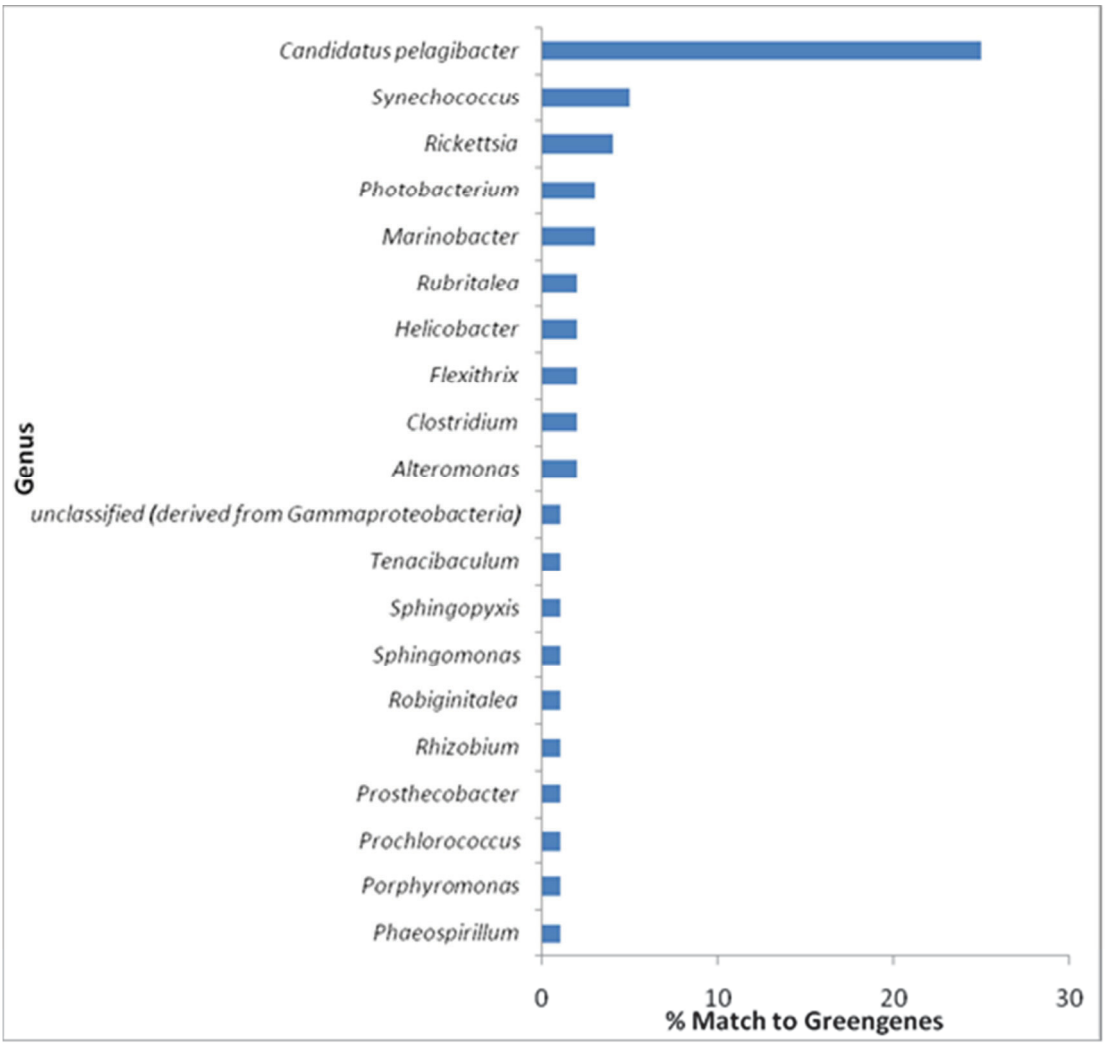
Network analysis

The resulting network displays the chemotactic OTUs as black nodes and the chemoattractants as coloured nodes, and it connects the OTUs to the chemoattractants they responded to via lines of the same colour as the chemoattractant. A coloured edge between an OTU and a chemoattractant indicates a chemotactic response by that OTU to that chemoattractant. The number of edges departing from an OTU then denotes the number of compounds which that OTU responds to. In cases where an OTU responded only to a single chemoattractant its corresponding node is connected only to that chemoattractant. In cases where an OTU responded to multiple chemoattractants, the corresponding node is connected to the relevant groups of chemoattractants.

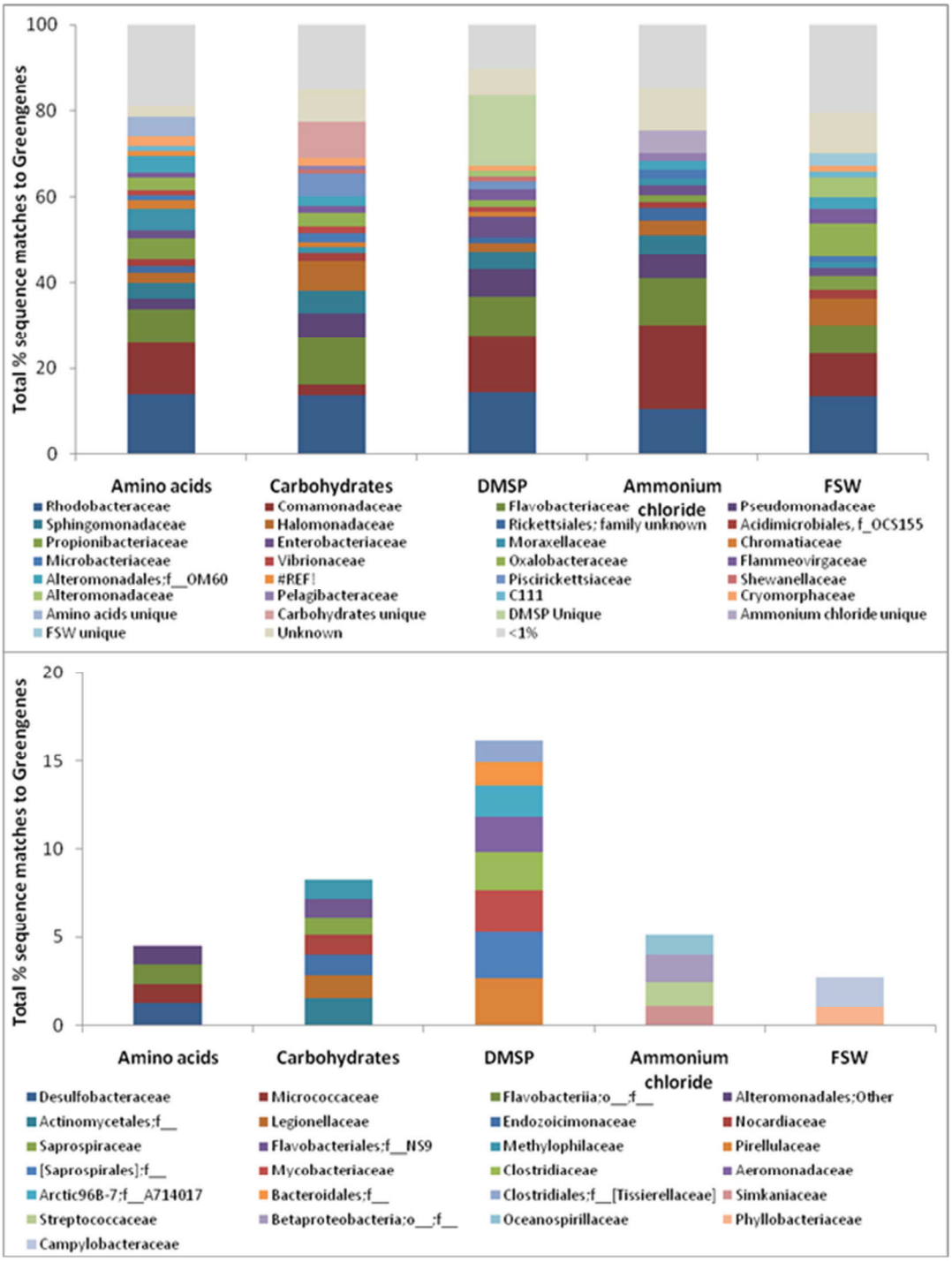


Supplementary figure S2.1 Map showing location of a) Heron Island on the Great Barrier Reef, Australia indicated by a red dot, and b) sample sites on Heron Island, the Great Barrier Reef where a) triangles depict sites where water was collected in July 2010 for laboratory experiments using syringe assays: blue depicts the non-coral associated site outside of the reef crest and red shows the coral-associated site on the surface of *Pocillopora damicornis* within the reef crest and b) circles represent microenvironments where *In Situ* Chemotaxis Assays (ISCAs) were deployed in July 2011 to collect both cell counts for flow cytometry and DNA for 16S rRNA amplicon pyrosequencing

Supplementary figure S2.2 Photograph depicting a ISCA prior to being fully inserted in between the branches of *Acropora palifera* in the lagoon, Heron Island.



Supplementary figure S2.3 Taxonomic identity (genus level) of the bacterial community inhabiting seawater collected from the surface of the coral species *Acropora aspera.* Relative representation in the metagenome was calculated by normalizing to total number of sequences. Taxonomy was assigned by using BLASTn to compare sequences to the Greengenes database in MG RAST. The composition of this bulk, background community differs substantially to the composition of the communities responding to chemoattractants in the ISCA deployment in this microenvironment.



Supplementary figure S2.4: a) Taxonomic identity of chemotactic bacteria determined using 16S rRNA tag pyrosequencing (not normalised to cell counts as represented in Fig. 2.3. Hits were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME. Chao1 diversity estimates revealed that carbohydrates had the highest diversity of all the chemoattractants at 236, followed by amino acids at 158.4, DSMP had a chao1 of 130, while ammonium chloride had a diversity of 125. b) Taxonomic identity of chemotactic bacteria unique to each chemoattractant (ie found in only that chemoattractant) as seen in Fig. 2.3 and Fig. 2.4a

Supplementary table S2.1: Two-way fixed factor ANOVA to determine chemotactic response using a modified capillary assay to DMSP at three concentrations in two water types (coral reef and non-coral reef). Table relates to data presented in Figure 2.1a i-ii.

Source of variation	d.f.	SS	MS	*F*	*P*
Water type	1	328.97117.56	328.9758.784	9.46	0.010*
Concentration	2	114.97	57.489	1.69	0.226
Water type x concentration	2			1.65	0.232
		417.42	34.785		
Error	12	987.94	3		
Total	17				

*= $P < 0.05$; ***= $P < 0.001$

Supplementary table S2.2: Three-way fixed factor ANOVA of the effects of water type (coral reef and non-coral reef), amino acid, and concentration on the chemotactic response of bacteria using a modified capillary assay. Table relates to data presented in Figure 2.1b i-ii .

Source of variation	d.f.	SS	MS	*F*	*P*
Water type	1	4.801	4.862	72.12	< 0.001***
Amino acid	5	1.120	0.225	3.34	0.009*
Concentration	2	0.364	0.178	2.64	0.078
Water type x amino acid	5	4.415	0.886	13.15	< 0.001***
Water type x concentration	2	0.111	0.049	0.74	0.480
Amino acid x concentration	10	1.321	0.127	1.88	0.062
Water type x amino acid x concentration	10	2.575	0.257	3.82	< 0.001***
Error	71	4.783	0.067		
Total	106	19.49			

*= $P < 0.05$; ***= $P < 0.001$

Supplementary table S2.3: Three-way fixed factor ANOVA on the effects of water type (coral reef and non-coral reef), carbohydrate, and concentration on the chemotactic response of bacteria using a modified capillary assay. Table relates to data presented in Figure 2.1c i-ii.

Source of variation	d.f.	SS	MS	*F*	*P*
Water type	1	14.417	10.320	14.08	< 0.001***
Carbohydrate	3	56.0345.8356	19.754	26.95	<0.001***
Concentration	2		2.594	3.54	0.038*
		37.94			
Water type x carbohydrate	3	0.281	12.91	17.62	<0.001***
Water type x concentration	2	1.742	0.147	0.20	0.818
Sugar x concentration	6		0.258	0.35	0.905
		7.2664			
Water type x carbohydrate x concentration	6		1.2111	1.65	0.156
		32.256			
Error	44	155.77	0.7331		
Total	67				

*= $P < 0.05$; ***= $P < 0.001$

Supplementary table S2.4: *Acropora aspera* reef crest ISCA: 2-Way fixed factor ANOVA to determine chemotactic response to the chemoattractants in the four sites (data log transformed).

Analysis of Variance for Ratio to FSW, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Site	3	598.111	598.225	199.408	29.79	0.000
Chemoattractant	5	216.261	394.633	78.927	11.79	0.000
Site*Chemoattractant	15	1046.462	1046.462	69.764	10.42	0.000
Error	77	515.442	515.442	6.694		
Total	100	2376.276				

S = 2.58729 R-Sq = 78.31% R-Sq(adj) = 71.83%

Supplementary table S2.5: Paired T-Test and CI for chemotactic responses to FSW and DMSP in ISCA deployed on *A. aspera* in the reef crest site (data log transformed).

	N	Mean	StDev	SE Mean
DMSP	3	1.4915	0.1377	0.0795
FSW	3	1.0000	0.0000	0.0000
Difference	3	0.4915	0.1377	0.0795

95% CI for mean difference: (0.1494, 0.8336)
T-Test of mean difference = 0 (vs not = 0): T-Value = 6.18 P-Value = 0.025

Supplementary table S2.6: Paired T-Test and CI for chemotactic responses to FSW and amino acids mix in ISCA deployed on *A. aspera* in the reef crest site (data log transformed).

	N	Mean	StDev	SE Mean
Amino acids mix	3	2.338	0.309	0.178
FSW	3	1.0000	0.0000	0.0000
Difference	3	1.338	0.309	0.178

95% CI for mean difference: (0.571, 2.106)
T-Test of mean difference = 0 (vs not = 0): T-Value = 7.50 P-Value = 0.017

Supplementary table S2.7: Paired T-Test and CI for chemotactic responses to FSW and ammonium chloride in ISCA deployed on *A. aspera* in the reef crest site (data log transformed).

	N	Mean	StDev	SE Mean
Ammonium chloride	3	1.5441	0.1183	0.0683
FSW	3	1.0000	0.0000	0.0000
Difference	3	0.5441	0.1183	0.0683

95% CI for mean difference: (0.2502, 0.8380)
T-Test of mean difference = 0 (vs not = 0): T-Value = 7.97 P-Value = 0.015

Supplementary table S2.8: Paired T-Test and CI for chemotactic responses to FSW and carbohydrate mix in ISCA deployed on *A. aspera* in the reef crest site (data log transformed).

	N	Mean	StDev	SE Mean
Carbohydrates mix	3	1.839	0.353	0.204
FSW	3	1.0000	0.0000	0.0000
Difference	3	0.839	0.353	0.204

95% CI for mean difference: (-0.038, 1.716)
T-Test of mean difference = 0 (vs not = 0): T-Value = 4.12 P-Value = 0.054

Supplementary Table S2.9: PRIMER table showing SIMPER analysis of metabolic categories between coral (lagoon-coral and reef crest-coral) and non-coral (open water and sandy substrate) metagenomes with an average dissimilarity of 7.19%. Genes associated with cell motility contributed to 46.09% of the differences between the coral-associated and non-coral associated samples.

	Group Coral-associated	Group Non-coral associated				
Cellular processes category	Average abundance	Average abundance	Average dissimilarity	Dissimilarity/SD	Contribution %	Cumulative %
Cell motility	4.96	3.82	3.31	1.24	46.09	46.09
Cell growth and death	7.05	7.67	1.72	1.46	23.88	69.98
Cell communication	1.48	0.99	1.36	3.91	18.98	88.95
Transport and catabolism	4.8	5.01	0.79	1.33	11.05	100

Supplementary Table S2.10: PRIMER table showing SIMPER analysis of metabolic categories between coral (lagoon-coral and reef crest-coral) and non-coral (open water and sandy substrate) metagenomes with an average dissimilarity of 13.72. Genes associated with bacterial chemotaxis contributed to 21.39% of the differences between the coral-associated and non-coral associated samples.

	Group Coral-associated	Group Non-coral associated				
Cell motility category	**Average abundance**	**Average abundance**	**Average dissimilarity**	**Dissimilarity/SD**	**Contribution %**	**Cumulative %**
02030 Bacterial chemotaxis [PATH:ko02030]	3.83	2.24	2.93	1.41	21.39	21.39
04113 Meiosis - yeast [PATH:ko04113]	1.05	0.22	1.56	1.12	11.38	32.77
04112 Cell cycle - Caulobacter [PATH:ko04112]	6.75	7.44	1.27	1.24	9.28	42.05
04540 Gap junction [PATH:ko04540]	1.19	0.61	1.07	3.23	7.77	49.82
04142 Lysosome [PATH:ko04142]	1.51	1.85	0.76	1.35	5.54	55.36
04520 Adherens junction [PATH:ko04520]	0.13	0.46	0.6	2.08	4.4	59.76
04115 p53 signaling pathway [PATH:ko04115]	1.11	1.43	0.59	1.18	4.32	64.08
04111 Cell cycle - yeast [PATH:ko04111]	0.2	0.48	0.57	1.35	4.16	68.24
02040 Flagellar assembly [PATH:ko02040]	3.14	3.04	0.53	1.21	3.85	72.09
04530 Tight junction [PATH:ko04530]	0.38	0.11	0.5	2.03	3.66	75.74
04810 Regulation of actin cytoskeleton [PATH:ko04810]	0.27	0	0.5	21.24	3.62	79.36
04210 Apoptosis [PATH:ko04210]	0.26	0.16	0.48	1.19	3.51	82.87
04114 Oocyte meiosis [PATH:ko04114]	0.24	0	0.45	0.87	3.25	86.12
04144 Endocytosis [PATH:ko04144]	1.04	0.99	0.37	2.06	2.73	88.85
04140 Regulation of autophagy [PATH:ko04140]	0.54	0.35	0.35	1.34	2.55	91.4

Supplementary Table S2.11: PRIMER table showing SIMPER analysis of metabolic categories between coral (lagoon-coral and reef crest-coral) and non-coral (open water and sandy substrate) metagenomes with an average dissimilarity of 34.58. Top cumulative 20% of proteins from the bacterial chemotaxis category within the KEGG database contributing to differences between the coral-associated and non-coral associated samples

	Group Coral-associated	Group Non-coral associated				
Bacterial chemotaxis category	**Average abundance**	**Average abundance**	**Average dissimilarity**	**Dissimilarity/SD**	**Contribution %**	**Cumulative %**
motB; chemotaxis protein MotB	1.21	0	0.78	8.06	2.26	2.26
cheY; two-component system, chemotaxis family, response regulator CheY	0.89	0	0.58	10.84	1.67	3.93
ftsW, spoVE; cell division protein FtsW	1.5	0.85	0.54	1.15	1.57	5.5
cheA; two-component system, chemotaxis family, sensor kinase CheA [EC:2.7.13.3]	1.37	0.65	0.54	1.17	1.56	7.06
mcp; methyl-accepting chemotaxis protein	2.27	1.45	0.54	1.36	1.56	8.63
E4.6.1.1; adenylate cyclase [EC:4.6.1.1]	0.82	0	0.53	0.86	1.54	10.17
cheR; chemotaxis protein methyltransferase CheR [EC:2.1.1.80]	0.8	0	0.52	2.75	1.51	11.67
cheW; purine-binding chemotaxis protein CheW	0.71	0	0.46	13.59	1.33	13.01
motA; chemotaxis protein MotA	1.05	0.48	0.42	1.19	1.22	14.22
pleD; two-component system, cell cycle response regulator	1.01	0.38	0.41	1.35	1.2	15.42
E2.7.1.36, MVK, mvaK1; mevalonate kinase [EC:2.7.1.36]	0.63	0	0.41	7.04	1.17	16.59
clpX, CLPX; ATP-dependent Clp protease ATP-binding subunit ClpX	2.12	2.71	0.39	2.19	1.12	17.72
E2.5.1.26, AGPS; alkyldihydroxyacetonephosphate synthase [EC:2.5.1.26]	0	0.57	0.37	2.42	1.08	18.79
CYC; cytochrome c	0.57	1.09	0.37	0.96	1.07	19.87
cheB; two-component system, chemotaxis family, response regulator CheB [EC:3.1.1.61]	0.82	0.37	0.36	1.32	1.04	20.9

Chapter 3:

Redefining the sponge-symbiont acquisition paradigm: Sponge microbes exhibit chemotaxis towards host-derived compounds

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Abstract

Marine sponges host stable and species-specific microbial symbionts that are thought to be acquired and maintained by the host through a combination of vertical transmission and filtration from the surrounding seawater. To assess whether the microbial symbionts also actively contribute to the establishment of these symbiosis, we performed *in situ* experiments on Orpheus Island, Great Barrier Reef, to quantify the chemotactic responses of natural populations of seawater microorganisms towards cellular extracts of the reef sponge *Rhopaloeides odorabile*. Significant levels of chemotaxis were observed (I_c = 5.7 ± 1.3, P<0.05) and 16S rRNA gene amplicon sequencing revealed that the phylogenetic composition of the chemotactic microbes was significantly different to seawater controls and included known 'sponge-specific' microbial phylotypes, including a cluster within the *Gemmatimonadetes* and another within the *Actinobacteria*. These findings establish a new mechanism for how sponges can acquire bacterial symbionts from the surrounding environment and reveal an active role of the symbionts in finding their host.

Key words: *Bacteria / Chemotaxis / Coral reefs / Sponge / Motility / Chemoattractant*

Introduction

Sponges contain microbial symbionts from all three domains of life – Bacteria, Archaea and Eukarya – which can comprise up to 35% of a sponge's biomass and are essential for host fitness and survival (reviewed in Webster and Taylor, 2012). Molecular surveys have revealed that many sponge-associated microbes occur exclusively within sponges (Hentschel *et al.*, 2002; Taylor *et al.*, 2007a) or are exceptionally rare in the surrounding coral reef environment (Taylor *et al*., 2013). These microbes often form monophyletic 'sponge-specific' 16S rRNA gene sequence clusters (Hentschel *et al.*, 2002; Taylor *et al.*, 2007a; Simister *et al*., 2012; Taylor *et al.*, 2013). Up to 55.5% of these previously identified sponge-specific sequence clusters were unable to be detected outside of sponges from an analysis of 12 million sequences (Taylor *et al.,* 2013), while up to 30% of sponge derived bacteria fall into Sponge Specific Sequence Clusters (Simister et al., 2012), further supporting the notion that these sequences are rare in the surrounding environment. The occurrence of these 'sponge-specific' bacteria in phylogenetically distant sponges from geographically isolated locations, coupled with the rarity of these bacteria in the surrounding environment, has led to interest in the evolutionary mechanisms that have maintained these complex and diverse symbioses (Webster et al., 2012b).

Previous research has indicated that sponges likely acquire their symbionts via the dual mechanisms of microbial filtration from the surrounding seawater (Reiswig, 1971; Webster et al., 2010) and vertical transmission from parent to offspring (Webster et al., 2010). However, it has remained unclear whether these microbial associations are solely controlled by the sponge or if the microbes are also capable of actively seeking out their hosts on coral reefs. For instance, it has recently been shown that coral-associated microbes display high levels of chemotaxis to chemicals released by the coral holobiont (Garren et al, 2014; Tout et al., 2015). Here we propose that chemotaxis may also be involved in the formation of 'sponge-specific' microbial interactions and we assessed this using the model Great Barrier Reef sponge *Rhopaloeides odorabile,* which hosts a highly diverse and stable microbial

community that is critical in regulating host health (Fan et al., 2013; Webster et al., 2001, 2008, 2011; Webster and Hill 2001). We assessed whether natural populations of coral reef bacteria exhibit chemotaxis towards cellular extracts of *R. odorabile* and examined whether previously identified sponge symbionts, which are rare or undetectable in seawater, are among these chemotactic microbes.

Methods

This study was conducted in July 2013 at Orpheus Island (18°35.5959'S, 146°28.9559'E), Great Barrier Reef. To assess microbial chemotaxis towards the sponge *R. odorabile*, we employed a microfluidic-based *in situ* chemotaxis assay (ISCA) (Tout et al. 2015).

ISCA

Soft lithography techniques (Whitesides et al., 2001; Seymour et al., 2008) were used to create a high-throughput method for the quantification of chemotaxis *in situ*. This *In Situ* Chemotaxis Assay (ISCA) consists of a matrix of 24 cylindrical wells embedded within a ~30 cm^2 slab of the soft, inert polymer polydimethylsiloxane (PDMS), allowing for the simultaneous and replicated testing of multiple chemoattractants, as well as on-chip controls. Each well has a diameter of 10 mm and a height of 1 mm, resulting in a volume of approximately 80 µL. Each well has two 1 mm diameter, 5 mm height holes connecting the interior of the chamber to the external environment. Individual chambers are filled with 80 µL of chemoattractants using a pipette. Over the course of the 4 h deployment, the chemoattractant diffuses into the external environment, creating a chemical gradient in the surrounding seawater (Berg et al., 1983) that triggers the migration of chemotactic bacteria into the wells.

ISCA deployment

Three replicate *R. odorabile* samples were collected and used to prepare cellular sponge extracts whereby the term cellular extract refers to the crude sponge

homogenate following filtration. A 1 cm^3 section of tissue was excised from each sponge and homogenised in 10 ml of 0.2 µm-filtered seawater using a mortar and pestle. The tissue homogenate was filtered through 0.2 µm syringe filters (Millipore) to remove microbial cells and subsequently filtered through Amicon Ultra 0.5 3 Kda filters (Millipore) to remove any remaining DNA. These sponge cellular extracts were used as a chemoattractant, along with filtered seawater (FSW) controls in three replicate ISCA deployments. We deployed a total of six ISCAs onto the Orpheus Island Pioneer Bay reef flat at 8 m water depth in a region devoid of sponges. For each ISCA deployment, 4 replicates each of the *R. odorabile* cellular extract and a 0.2 µm-filtered seawater (FSW) control were randomly distributed across the ISCA. Background seawater samples were also collected from the same location prior to ISCA deployments using 1 L Schott bottles. Three ISCAs and the background seawater sample were subsequently used for flow cytometry to obtain total cell counts and the other three were used for obtaining DNA extracts for assessment of community composition using 16S rRNA gene amplicon pyrosequencing.

Chemotaxis Sample Preparation and Analysis

The relative strength of chemotactic response was determined using flow cytometry (FCM), whereby the number of cells that migrated into the ISCA wells was quantified (Tout et al., 2015). Upon completion of the assays, samples were immediately (within 10 min) fixed with glutaraldehyde (1% final concentration) and frozen in liquid nitrogen, before being stored at -80°C. Prior to FCM analysis, samples were quick-thawed, stained with SYBR Green I [1:10,000] (Invitrogen Molecular Probes USA), and 1 µm diameter fluorescent microspheres (Invitrogen Molecular Probes USA) were added as an internal reference (Marie et al., 1997). Samples were analysed using a Becton Dickinson LSR II flow cytometer and bacterial populations were discriminated from background particles according to SYBR Green fluorescence and side-scatter (Marie et al., 1997) using Cell-Quest Pro data analysis software (BD Biosciences) (Seymour et al., 2005, 2007; Tout et al., 2015). The accumulation of bacteria in response to the chemoattractants was expressed in terms of a Chemotactic Index, I_c (mean ± standard deviation) (Tout et al. 2015). The I_c value was calculated as the number of cells responding to the

chemoattractant relative to the number of cells responding to the FSW control using flow cytometry (Tout et al., 2015).

DNA Extraction, 16S rRNA Gene Sequencing and Analysis

ISCA retrieval and background seawater collection, DNA samples were quick-frozen in liquid nitrogen and stored at -80°C. Prior to extraction, DNA samples were quick-thawed in a bath of hot water and the entire volume from each sample was transferred into individual microfuge tubes. In the case of the background seawater sample, 1 mL was used. Microfuge tubes were spun at 14,000 rpm in a microcentrifuge for 10 min to pellet microbial cells. Genomic DNA was extracted from the pelleted samples using the Ultra Clean Microbial DNA extraction kit (Mo Bio, Carlsbad, California, USA). The four replicate samples of each chemoattractant across the three ISCAs designated for DNA collection were pooled to account for biological variability. The composition of the microbial communities responding to the sponge cellular extract was determined using 16S rRNA gene amplicon pyrosequencing. Extracted DNA was amplified using the 16S primers 803F (5'-ATTAGATACCCTGGTAGTC-3') and 1392R (5'-ACGGGCGGTGTGTRC-3') under the following cycling conditions: 95°C for 3 min; 25 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 90 s; followed by a final extension at 72°C for 10 min (Engelbrektson et al., 2010). Amplicons were sequenced using the 454 GS-FLX pyrosequencing platform (Roche) at the Australian Centre for Ecogenomics (University of Queensland, Australia).

16S rRNA gene sequences were analysed using the QIIME pipeline (Caporaso et al., 2010; Kuczynski et al., 2011). Briefly, DNA sequences were de-multiplexed and reads shorter than 200 bp, with a quality score <25 or containing homopolymers exceeding 6 bp, were discarded. Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar et al., 2010) and taxonomy was assigned against the Greengenes database (version 13_5; McDonald et al., 2012) using BLAST (Altschul et al., 1990). Chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011) and filtered from the dataset and sequences were rarefied. The abundance of each family in every sample was multiplied by the

bacterial cell abundance from the FCM data in the same sample to normalise the taxonomic composition according to cell abundance. A representative sequence of each identified OTU was taxonomically assigned (using a BLAST search) with a curated SILVA 16S rRNA database containing 173 previously identified bacterial sponge-specific clusters (SC) and 32 sponge/coral-specific clusters (SCC) (Simister et al. 2012). For each BLAST search, the 10 best hits were aligned in order to determine sequence similarities. A sequence was assigned to an SC/SCC when it was more similar to the sequences comprising that cluster than to other sequences outside the cluster and the similarity to this sequence was at least 75% (Taylor et al. 2013).

Statistical Analysis of Data

Positive chemotaxis was defined as occurring when the Chemotactic Index I_c was significantly ($P < 0.05$) greater than 1. I_c data were tested for normality using the Kolmogorov-Smirnov test and Levene's test was used to test for homogeneity of variance. A 1-way ANOVA was used to determine differences between the FSW control and the sponge cellular extract (FSW*cellular extract). All analyses were performed using Minitab software (version 15.1.0.0 2006, Pennsylvania, USA). Multivariate statistical software (PRIMER v6) was used to measure the degree of similarity between the community composition of the cellular extract, FSW control and background samples (Clarke and Gourley, 2006). Data was square-root transformed and the Bray-Curtis similarity was calculated between samples. SIMPER analysis (Clarke, 1993) was used to identify the taxa contributing most to the dissimilarity between the cellular extract, FSW control and background samples.

Results

Across all ISCAs the chemotactic response of the natural microbial community was significantly greater to the cellular extract of *R. odorabile* than to the FSW control ($I_c = 5.7 \pm 1.3$, $P<0.05$; Fig. 3.1, Table 3.1). The composition of the chemotactic bacteria responding to *R. odorabile* extracts was also different to the community of bacteria responding to the FSW control (Fig. 3.2a, Fig. 3.3) with an overall community dissimilarity of 53% (Table 3.2). The most abundant bacterial families

responding to cellular extracts of *R. odorabile* were the *Sphingomonadaceae, Rhodobacteraceae* and *Piscirickettsiaceae*, which represented 12%, 9% and 8% of the total community respectively (Fig. 3.2a, Fig. 3.3, Table 3.3). The primary drivers of difference between the community responding to *R. odorabile* extracts and the FSW control according to SIMPER analysis were members of the *Piscirickettsiaceae, Sphingomonadaceae*, and *Vibrionaceae* (each contributed 2% to the total dissimilarity), which were all more abundant in the *R. odorabile* cellular extracts than in the FSW controls (Fig. 3.2a, Fig. 3.3,Table 3.2). Among the chemotactic bacteria from both the *R. odorabile* cellular extracts and the FSW controls, we found 56 sequence matches to previously defined 'sponge-specific' sequence clusters (Table 3.4). Of these, 96% occurred in the *R. odorabile* cellular extracts (Fig. 3.2b), with 66% affiliated to the *Gemmatimonadetes* cluster SC67 (defined by Simister et al. 2012) and 30% to the *Actinobacteria* cluster SC22 (Fig. 3.2b).

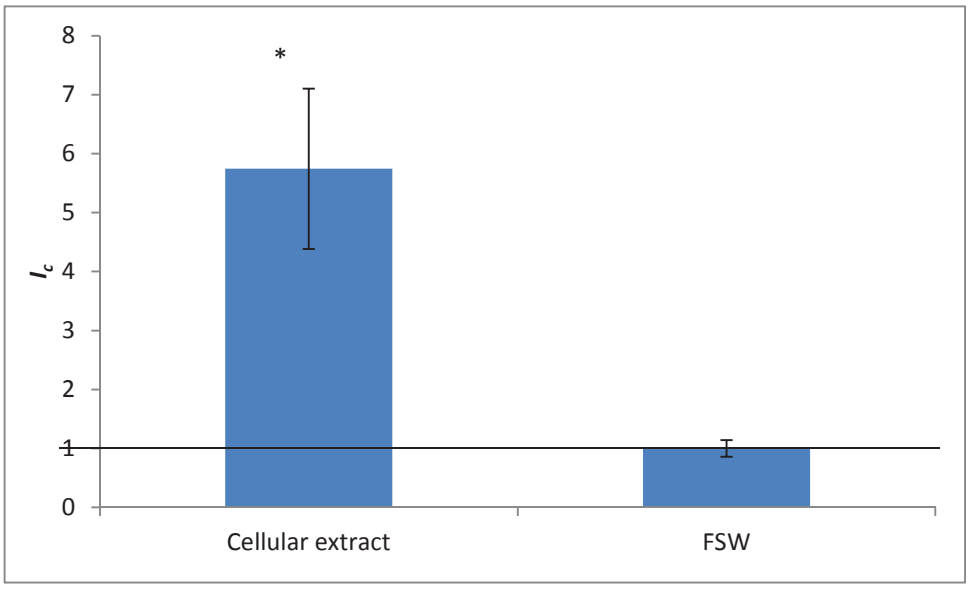

Figure 3.1 Bacterial chemotaxis to cellular extracts of the sponge *Rhopaloeides odorabile* observed at Orpheus Island using the *In Situ* Chemotaxis Assay (ISCA). I_c is the chemotactic index and responses above $I_c = 1$ represent positive chemotaxis. Vertical bars represent mean ± SD ($n = 4$). An asterisk represents chemotaxis that is significantly higher than the FSW control; α=0.05.

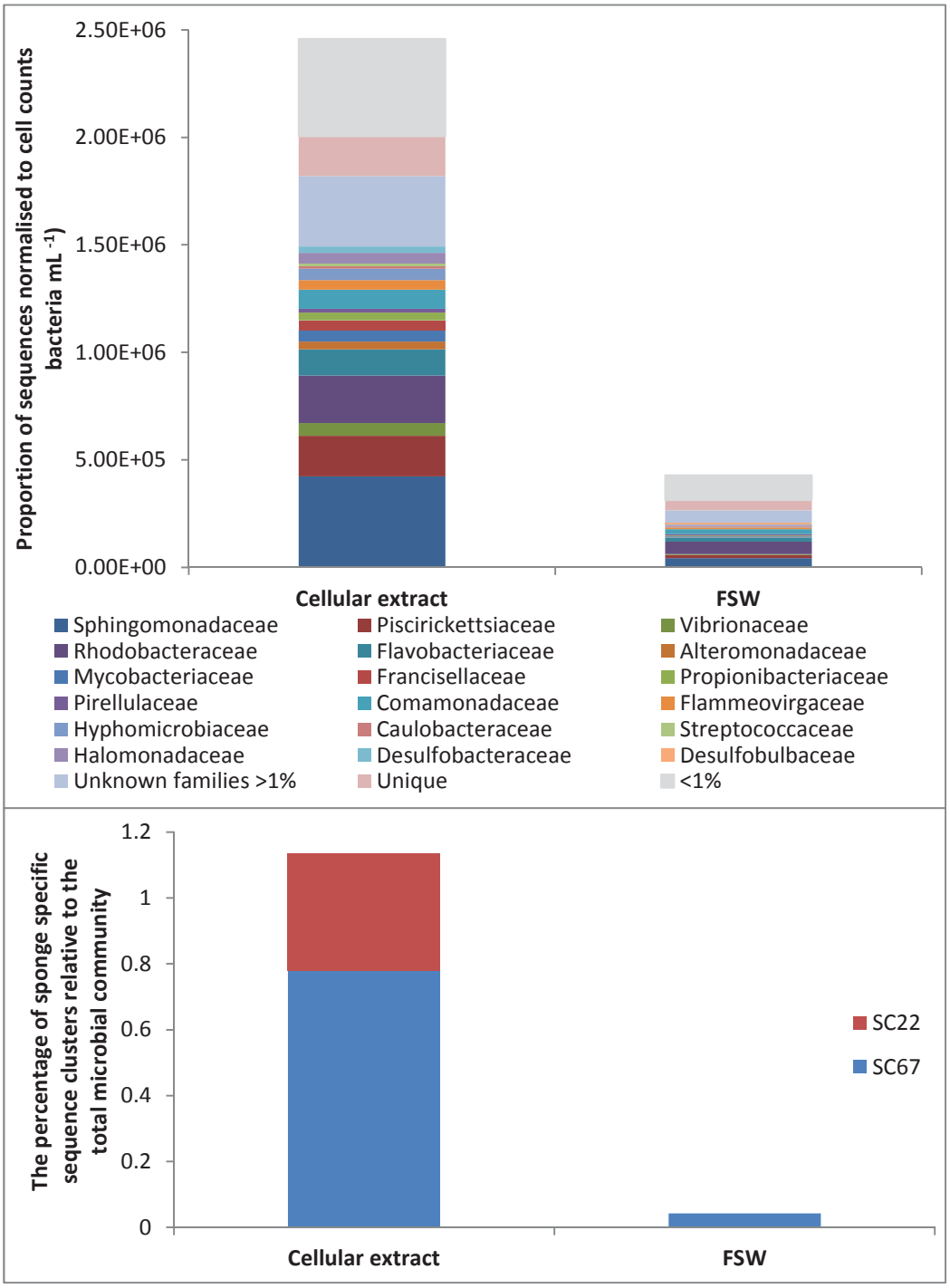

Figure 3.2 a) Taxonomic composition of bacteria at the family level responding to the ISCA deployments (data are averages of *n*=3 treatment) as determined by 16S rRNA gene sequencing. Hits were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME. The relative proportions of families responding to each chemoattractant were normalised to cell abundance retrieved from FCM (Fig. 3.1) (Dennis et al., 2013; Tout et al., 2015). The microbial community identified in the FSW control is representative of organisms that swam into this treatment as a consequence of random motility, rather than chemotaxis due to the lack of any chemical gradient. Thus, this sample provides an overview of the motile, but not necessarily chemotactic proportion of the community. **b)** Composition of chemotactic 'sponge-specific' sequence clusters (SC) in the

cellular extract of *R. odorabile* and FSW control generated by screening obtained sequences against a curated SILVA 16S rRNA database containing previously identified SC (Simister et al., 2012), as described in Taylor et al., (2013).

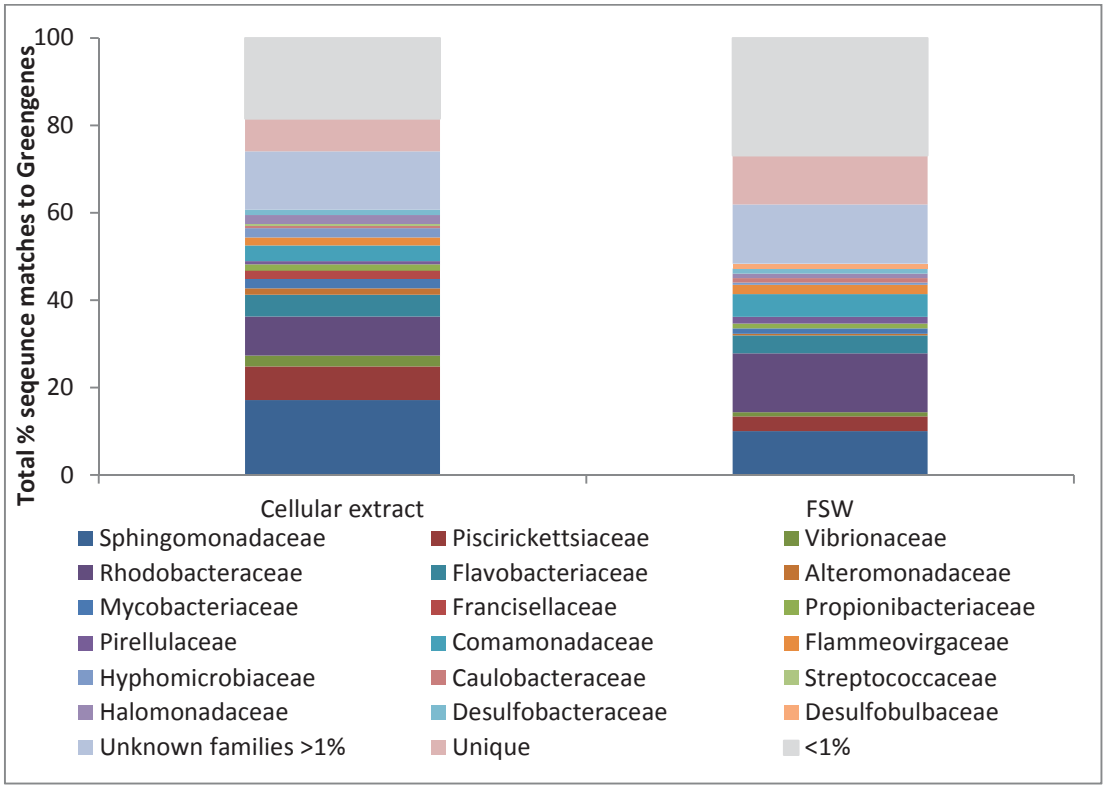

Figure 3.3: Taxonomic identity of chemotactic bacteria determined using 16S rRNA tag pyrosequencing (not normalised to cell counts as represented in Fig. 3.2a. Hits were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME.

Table 3.1: One-way fixed factor ANOVA to determine chemotactic response using an *In Situ* Chemotactic Assay (ISCA) containing cellular extracts of *R. odorabile* and FSW control. Table relates to data presented in figure 1.

Source of variation	**d.f.**	**SS**	**MS**	***F***	***P***
Cellular extract of *R. odorabile* x FSW	1	33.74	33.74	12.01	0.026*
Error	4	11.24	2.81		
Total	5	44.97			

*= $P < 0.05$; S = 1.676; R-Sq = 75.02%; R-Sq(adj) = 68.77%

Table 3.2 PRIMER table showing SIMPER analysis of chemotactic families contributing to dissimilarities between the cellular extracts of *R. odorabile* and the FSW control used in ISCA deployments on Orpheus Island with an average dissimilarity of 52.43%.

Groups Cellular extract & FSW						
Average dissimilarity = 52.43						
Species	Group Homogenate Av.Abund	Group FSW Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Piscirickettsiaceae	0.27	0.15	1.1	1.17	2.1	2.1
Sphingomonadaceae	0.41	0.3	1.05	1.74	2.01	4.11
Francisellaceae	0.11	0	0.81	1.32	1.55	5.66
Vibrionaceae	0.12	0.08	0.79	1.34	1.5	7.16
Rhizobiales; unknown	0	0.1	0.75	4.83	1.42	8.58
Alteromonadaceae	0.11	0.04	0.69	1.45	1.31	9.9
Halomonadaceae	0.13	0.09	0.65	1.24	1.24	11.13
Deltaproteobacteria; unknown	0	0.08	0.63	2.06	1.21	12.34
Hyphomicrobiaceae	0.14	0.06	0.63	1.3	1.2	13.54
Pirellulaceae	0.06	0.13	0.63	1.62	1.19	14.74
Caulobacteraceae	0.04	0.11	0.62	1.47	1.19	15.93
Gammaproteobacteria; o__HOC36	0.09	0.03	0.61	1.29	1.17	17.1
Gammaproteobacteria; unknown	0.09	0.1	0.6	1.12	1.15	18.25
Alteromonadales; unknown	0.08	0.07	0.6	1.4	1.14	19.39
Comamonadaceae	0.19	0.22	0.6	2.68	1.14	20.53
Saprospiraceae	0.1	0.04	0.59	1.49	1.12	21.66
Desulfobulbaceae	0.03	0.09	0.57	1.81	1.08	22.74
Acidobacteria;c__Sva0725	0.08	0.07	0.55	1.34	1.05	23.79
Rhodobacteraceae	0.3	0.36	0.55	1.16	1.04	24.83
Desulfobacteraceae	0.09	0.09	0.54	1.14	1.03	25.87
Alteromonadales; unknown	0	0.07	0.54	1	1.03	26.9
Colwelliaceae	0	0.07	0.54	1.04	1.02	27.92
Moraxellaceae	0.03	0.07	0.52	1.1	1	28.92
Acidimicrobiales;f__OCS155	0.07	0.09	0.51	1.29	0.98	29.9
Propionibacteriaceae	0.1	0.1	0.5	1.3	0.95	30.86

Table 3.3 PRIMER table showing SIMPER analysis of chemotactic families responding to the cellular extract of *R. odorabile* with an average similarity of 49.66% across all three ISCA deployments on Orpheus Island (n=4)

Group Cellular Extract					
Average similarity: 49.66					
Species	**Av.Abund**	**Av.Sim**	**Sim/SD**	**Contrib%**	**Cum.%**
Sphingomonadaceae	0.41	5.91	8.99	11.9	11.9
Rhodobacteraceae	0.3	4.27	4.37	8.61	20.51
Piscirickettsiaceae	0.27	3.86	16.56	7.76	28.27
Flavobacteriaceae	0.22	3.3	6.28	6.64	34.91
Alteromonadales;f__OM60	0.2	2.96	23.92	5.95	40.87
Comamonadaceae	0.19	2.67	4.45	5.38	46.25
Alphaproteobacteria;o__;f__	0.17	2.39	4.48	4.81	51.06
Mycobacteriaceae	0.15	2.03	4.82	4.08	55.14
Hyphomicrobiaceae	0.14	2.01	13.23	4.05	59.19
Chromatiales;f__	0.12	1.49	5.66	3	62.19
Halomonadaceae	0.13	1.4	1.32	2.82	65.01
Unassigned	0.09	1.16	20.53	2.34	67.35
Acidimicrobiales;f__	0.1	1.1	6.68	2.21	69.56
Saprospiraceae	0.1	1.09	2.1	2.19	71.74
Alteromonadaceae	0.11	1.08	2.4	2.17	73.92
Cryomorphaceae	0.08	1.02	2.78	2.05	75.97
Francisellaceae	0.11	0.84	0.58	1.69	77.65
Acidimicrobiales;f__C111	0.07	0.84	1.73	1.68	79.33
Flammeovirgaceae	0.09	0.67	0.58	1.34	80.67
Gammaproteobacteria;o__HOC36;f__	0.09	0.65	0.58	1.31	81.99
Gammaproteobacteria;o__;f__	0.09	0.65	0.58	1.3	83.29
Propionibacteriaceae	0.1	0.63	0.58	1.27	84.56
Vibrionaceae	0.12	0.62	0.58	1.26	85.82
Desulfobacteraceae	0.09	0.58	0.58	1.16	86.98
Rhodospirillaceae	0.08	0.55	0.58	1.1	88.08
Alteromonadales;f__	0.08	0.5	0.58	1	89.09
Acidimicrobiales;f__OCS155	0.07	0.48	0.58	0.97	90.06

Table 3.4 Table showing previously identified sponge-specific sequence clusters that were detected among the OTUs in this study; taxonomy matches were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME.

OTU	Sample	Hits	Sponge Specific Cluster (SC) or Sponge and Coral-Specific Cluster (SCC)	Taxonomy
denovo 532	Cellular Extract 2	11	SC67	Gemmatimonadetes
denovo 532	Cellular Extract 3	25	SC67	Gemmatimonadetes
denovo 561	Cellular Extract 3	1	SC67	Gemmatimonadetes
denovo 619	Cellular Extract 3	17	SC22	Actinobacteria
denovo 311	FSW	2	SC67	Gemmatimonadetes

Discussion

The important role of chemotaxis in structuring host-bacterial associations within the marine environment is becoming increasingly apparent (Banin et al., 2001; Rosenberg et al., 2007; Meron et al., 2009; Garren et al., 2014; Tout et al., 2014). Here we present the first observations of chemotaxis towards sponge-derived compounds, measured *in situ* with natural populations of coral reef bacteria. Importantly, previously defined 'sponge-specific' bacteria were not detected by amplicon sequencing of the background coral reef seawater, which is consistent with previous reports that these microorganisms are exceptionally rare outside of sponge hosts (Taylor et al., 2013). However, some environmentally rare 'sponge-specific' bacteria did exhibit chemotaxis towards sponge-derived chemicals, highlighting a new mechanism for the establishment of sponge-bacteria associations.

The concept of sponges having specific and stable symbionts that provide benefit to their hosts was originally proposed by Vacelet and Donadey (1977) and subsequently validated by extensive molecular research (reviewed in Webster and Taylor, 2012; Hentschel et al., 2012). For example, members of the *Piscirickettsiaceae* and *Sphingomonadaceae*, which were taxa primarily responsible for driving differences

in the communities responding to the sponge chemoattractant and the FSW control in the present study, are known to form intimate associations with marine sponges (Thomas et al., 2010) and have been linked by functional metagenomics to the production of enzymes involved in the vitamin B12 synthesis pathway (Thomas et al. 2010).

The theory of sponge symbiont specificity was expanded by the discovery that sponges host microorganisms falling within monophyletic 'sponge-specific' 16S rRNA gene sequence clusters (Hentschel *et al.*, 2002; Taylor *et al.*, 2007a; Simister *et al*., 2012; Taylor *et al.*, 2013). These 'sponge-specific' sequence clusters are defined as groups of 16S rRNA gene sequences that share greater similarity to each other than to sequences from non-sponge sources, are derived from at least two or more sponge species (or the same species from at least two different locations) and are supported by at least three independent phylogenetic tree-building algorithms (Hentschel et al., 2002).

The observed chemotaxis of microbes within the *Gemmatimonadetes* and *Actinobacteria* 'sponge-specific' 16S rRNA gene sequence clusters therefore provides further evidence that some sponge symbionts may actively find their sponge host on coral reefs using chemotaxis. These microbial phyla are known to associate with a diverse range of sponges (Taylor et al. 2007) and are highly active within their respective hosts (Kamke et al. 2010), yet have been shown to be rare in the surrounding seawater on the Great Barrier Reef (Webster et al., 2010; Bourne et al., 2013).

This study clearly illustrates that chemotaxis may underpin the establishment of some sponge-bacterial associations. Future studies that further refine the specific chemical cues derived from different sponge hosts are likely to reveal further patterns of sponge symbiont attraction. These observations indicate that the behaviour of individual microbes can underpin the establishment of an important animal-microbe symbiosis and adds to the growing evidence that chemotaxis is an ecologically important phenotype with the marine environment.

Chapter 4:

Increased seawater temperature increases the abundance and alters the structure of natural *Vibrio* populations associated with the coral *Pocillopora damicornis*

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Abstract

Rising seawater temperature associated with global climate change is a significant threat to coral health and is linked to increasing coral disease and pathogen-related bleaching events. We performed heat stress experiments with the coral *Pocillopora damicornis*, where temperature was increased to 31° C, consistent with the 2–3° C predicted increase in summer sea surface maxima. 16S rRNA amplicon sequencing revealed a large shift in the composition of the bacterial community at 31° C, with a notable increase in *Vibrio*, including known coral pathogens. To investigate the dynamics of the naturally occurring *Vibrio* community, we performed quantitative PCR targeting (i) the whole *Vibrio* community and (ii) the coral pathogen *Vibrio coralliilyticus*. At 31° C, *Vibrio* abundance increased by 2–3 orders of magnitude and *V. coralliilyticus* abundance increased by four orders of magnitude. Using a *Vibrio*-specific amplicon sequencing assay, we further demonstrated that the community composition shifted dramatically as a consequence of heat stress, with significant increases in the relative abundance of known coral pathogens. Our findings provide quantitative evidence that the abundance of potential coral pathogens increases within natural communities of coral-associated microbes as a consequence of rising seawater temperature and highlight the potential negative impacts of anthropogenic climate change on coral reef ecosystems.

Keywords: *Vibrio*, *Vibrio coralliilyticus*, *Pocillopora damicornis*, corals, heat stress, pathogen

Introduction

The health and function of coral reefs is profoundly influenced by microorganisms, which often form species-specific associations with corals (Rohwer et al., 2002; Rosenberg et al., 2007; Mouchka et al., 2010). These ecological relationships can be mutualistic, commensal or pathogenic (Rosenberg et al., 2007), and diseases caused by pathogenic microbes have been identified as a key threat to coral reefs globally (Bourne et al., 2009; Burge et al., 2014). Diseases including white syndrome – which causes bleaching and lysis (Kushmaro et al., 1996; Ben-Haim et al., 2003a; Rosenberg and Falkovitz, 2004), white band (Ritchie and Smith, 1998; Aronson and Precht, 2001), white plague (Thompson et al., 2001), white pox (Patterson et al., 2002), black band (Frias-Lopez et al., 2002; Sato et al., 2009), and yellow band (Cervino et al., 2008) have all been attributed to microorganisms and have led to mass mortalities and significant loss of coral cover (Bourne et al., 2009).

There is evidence that the occurrence and severity of coral disease outbreaks is increasing globally (Harvell et al., 2004; Bruno et al., 2007; Mydlarz et al., 2010), potentially due to environmental stressors associated with phenomena such as increases in seawater temperature (Mouchka et al., 2010; Ruiz-Morenol et al., 2012). Heat stress may compromise the health of corals, leading to enhanced susceptibility to disease (Hoegh-Guldberg, 1999; Hoegh-Guldberg and Hoegh-Guldberg, 2004; Jokiel and Brown, 2004), or increase the abundance and/or virulence of pathogens (Vega Thurber et al., 2009; Vezzulli et al., 2010; Kimes et al., 2011). Increases in seawater temperature have been shown to change the composition and functional capacity of coral-associated microbial communities, including shifts to an elevated state of virulence, and pathogenicity (Vega Thurber et al., 2009).

While diverse groups of microbes, including bacteria, fungi, and viruses have been implicated in several coral diseases, one bacterial genus in particular has become a recurrent feature within coral disease research. *Vibrio* are globally distributed marine *Gammaproteobacteria* (Pollock et al., 2010), which habor a diverse virulence repertoire that enables them to be efficient and widespread pathogens of a wide range of marine species (Santos Ede et al., 2011), including shellfish (Jeffries, 1982), fish

(Austin et al., 2005), algae (Ben-Haim et al., 2003b), mammals (Kaper et al., 1995; Shapiro et al., 1998; Oliver, 2005), and corals (Ben-Haim et al., 2003b). White syndrome in *Montipora* corals is caused by *V. owensii* (Ushijima et al., 2012), white band disease II in *Acropora cervicornis* has been attributed to *V. charchariae* (synonym for *V. harveyi*; Gil-Agudelo et al., 2006; Sweet et al., 2014), and a consortium of *Vibrio* are responsible for yellow band disease (Cervino et *al.,* 2008; Ushijima et al., 2012). Furthermore, *V. shiloi* and *V. coralliilyticus* are the causative agents of bleaching in the coral species *Oculina patagonica* (Kushmaro et *al.,* 1996, *1998*; Toren et al., 1998) and the cauliflower coral *Pocillopora damicornis* (Ben-Haim and *Rosenberg,* 2002; Ben-Haim et *al.,* 2003b), respectively.

Laboratory experiments using cultured isolates of *V. shiloi* (Kushmaro et al., 1996, 1997) and *V. coralliilyticus* (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b) have fulfilled Koch's postulates, with each species proven to be the causative agent of coral bleaching. *V. shiloi* causes bleaching in *O. patagonica* by using chemotaxis toward the coral mucus, before adhering to the coral surface and penetrating the epidermis (Banin et al., 2001). After colonization of the coral, cell multiplication occurs followed by production of the Toxin P molecule, which inhibits photosynthesis in the symbiotic zooxanthellae, resulting in coral bleaching, and tissue loss (Rosenberg and Falkovitz, 2004). Similarly, *V. coralliilyticus* causes bleaching, lysis and tis-sue loss in the coral *P. damicornis* (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b; Meron et al., 2009; Garren et al., 2014). The mechanism behind *V. coralliilyticus* infection also includes motility and chemotaxis (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b) and involves the post-colonization production of a potent extracellular metalloproteinase, which causes coral tissue damage (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b). Another key similarity in the infection and bleaching mechanisms of *V. shiloi* and *V. coralliilyticus* is an increased infection rate under elevated seawater temperatures (Toren et al., 1998; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b).

Heat stress can enhance coral disease by increasing host susceptibility to infection by pathogens (Bourne et al., 2009; Mouchka et al., 2010) or altering the behaviour and

virulence of pathogenic bacteria (Kushmaro et al., 1998; Banin et al., 2001; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003a,b; Koren and Rosenberg, 2006; Bourne et al., 2008; Kimes et al., 2011; Santos Ede et al., 2011). Notably, *V. shiloi* can only be isolated from bleached corals during summer months (Kushmaro et al., 1998) and laboratory experiments have shown that this species causes bleaching at an accelerated rate above 29°C, yet has negligible effect at 16°C (Kushmaro et al., 1998). Similarly, tissue loss caused by *V. coralliilyticus* is most rapid at elevated temperatures between 27 and 29°C (Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b). Seawater temperatures above 27°C have also been shown to play a direct role in the up-regulation of several *V. coralliilyticus* virulence genes, including factors involved in host degradation, secretion, antimicrobial resistance, and motility (Kimes et al., 2011). Up-regulation of motility is particularly notable as both *V. shiloi* and *V. coralliilyticus* exhibit enhanced chemotactic capacity at elevated temperatures (Banin et al., 2001; Garren et al., 2014). Heat-stressed corals also increase the production and release of signaling compounds including dimethylsulfoniopropionate (DMSP) at elevated temperature, further enhancing the ability of pathogens to locate, and colonize heat-stressed corals (Garren et al., 2014).

To date, our understanding of coral-associated *Vibrio* dynamics under elevated seawater temperatures has been solely derived from laboratory-based experiments using cultured isolates (Kushmaro et al., 1998; Toren et al., 1998; Banin et al., 2001; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b; Garren et al., 2014). However, there is currently little understanding of how native communities of *Vibrio*, occurring within diverse natural assemblages of bacteria, will respond to elevated seawater temperatures. Understanding the dynamics of *Vibrio* populations within this complex, but also more realistic, scenario is important because it is very probable that Vibrios living in co-habitation with other competing and interacting species, will display different dynamics to those displayed by cultured isolates under laboratory conditions. For instance, interspecies antagonistic interactions among bacteria can strongly influence the growth and proliferation of other *Vibrio* species (Long et al., 2005), and we may expect similar ecological complexities to also occur within the coral holobiont. Here, we examined changes in the *Vibrio* population within a natural, mixed community of bacteria associated with the coral species *P.*

damicornis on Heron Island, the Great Barrier Reef, Australia, and demonstrate that heat stress increases the abundance and changes the composition of potentially pathogenic *Vibrio* populations associated with corals.

Materials and Methods

Heat Stress Experiment

Three separate colonies (denoted A, B, and C) of the coral species *P. damicornis* were collected from within the Heron Island lagoon, on the Great Barrier Reef, Australia (23° 26'41'' S, 151° 54'47'' E), and translocated to the Heron Island Research Station. Colonies were placed into flow-through aquaria, which circulated water pumped from the reef flat to the Heron Island Research Station. The colonies were fragmented into 48 nubbins using bone cutters and acclimated for 8 days across six flow-through experimental tanks. The placement of the nubbins from each colony within each tank and the position of the tanks were randomized. During the experiment, three tanks were maintained at the ambient seawater temperature (22° C) experienced on the reef flat (control), while the remaining three tanks were exposed to a heat stress treatment, which involved the incremental ramping of seawater temperature by 1.5°C each day for seven consecutive days using one 25W submersible aquarium heater (Aqua One, Ingleburn, NSW, Australia) per tank, until a final temperature of 31°C was reached. Water was circulated in the tanks using one 8W maxi 102 Powerhead pump (Aqua One, Ingleburn, NSW, Australia) per tank. This temperature increase is in line with the predicted 2–3°C increases above current summer average seawater temperature (Hoegh-Guldberg, 1999, 2004; Berkelmans et al., 2004; Hoegh-Guldberg et al., 2007) for Heron Island. Coral nubbins were sampled using sterile forceps at the start of the experiment (t_0) and after 7 days for both the control (t_{final} Control) and heat stress treatments (t_{final} Heat stress). The nubbins were immediately placed into 15 mL falcon tubes containing 3 mL of RNA*later* (Ambion, Life Technologies, Australia; Vega Thurber et al., 2009), which was a sufficient volume to completely immerse the nubbins. The nubbins were subsequently stored at −80°C until processing.

Photosynthetic Health of Corals

Photosynthetic health of the corals was checked using a diving pulse amplitude modulated (PAM) fluorometer (Walz, Germany) in the t_{final} Control and t_{final} 31° C treatments. Corals were dark-adapted for 10 min before their minimum fluorescence in the dark (F_O) was recorded. Maximum fluorescence (F_M) was determined using a saturating pulse of light for 0.8 s. The corals were then illuminated under 616 µmol photon m^{-2} s^{-1} light for 5 min to test their ability to sustain photosynthetic function under light. Maximum Quantum Yield (F_V/F_M) was measured on dark-adapted samples and effective quantum yield Y(PSII), regulated non-photochemical quenching Y(NPQ), and non-regulated non-photochemical quenching Y(NO) were measured on light adapted samples. To compare the changes in the F_V/F_M, Y(PSII), Y(NPQ), and Y(NO) measurements in the t_0, t_{final} Control, and t_{final} Heat Stress treatments, a 1-way analysis of variance (ANOVA) was used (treatment) to determine significant differences ($P < 0.05$) between these measurements. Prior to this, data was tested for normality using the Kolmogorov– Smirnov test and Levene's test was used for homogeneity of variance.

Coral-Bacterial Cell Separation

Coral nubbins were thawed slowly on ice and removed from the RNA-later solution using sterile forceps and kimwipes to remove excess solution (Vega Thurber et al., 2009). Replicate nubbins from the same donor colony (A, B, or C) were pooled and placed into sterile 150 mL conical flasks containing 15 mL sterile-autoclaved calcium and magnesium free seawater plus 10 mM EDTA (CMFSWE). The surfaces of the nubbins were airbrushed using 80 psi with a sterile 1 mL barrier tip (fresh tip for each new nubbin) in the conical flasks using sterile forceps to hold the nubbin in place. For each sample, the 15 mL tissue slurry was then filtered through a sterile 100 µm cell strainer (BD 352360) into a sterile 50 mL plastic centrifuge tube to remove host cells. The <100 µm filtrate was then filtered through a 3 µm filter (Whatman) and sterile filter tower apparatus (Nalgene) using vacuum pressure to remove any host cells larger than 3 µm. The resultant <3 µm filtrate (~15 mL) was

centrifuged at 14462 × *g* to pellet the microbes for 5 min. DNA was extracted from the cell pellet using the MO BIO Ultra Clean Microbial DNA Kit (Carlsbad, CA, USA) according to the manufacturer's instruc-tions. Genomic DNA concentrations were measured using a Qubit 2.0 fluorometer (Invitrogen).

16S rRNA Amplicon Sequencing and Analysis

The bacterial community composition in each nubbin was deter-mined using the universal bacterial 16S rRNA gene primers 27F (5' - AGAGTTTGATCMTGGCTCAG-3') and 1392R (5'- ACGGGCGGTGTGTRC-3'; resulting in a 1365bp product) and the HotStarTaq Plus Master Mix Kit (Qiagen, USA). A 30 cycle amplification process was employed, incorporating the following cycling conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72° C for 1 min, after which a final elongation step at 72° C for 5 min was performed. In addition, the composition and diversity of the *Vibrio* community was assessed using the *Vibrio* specific 16S rRNA gene primers VF169 (5' - GGATAACYATTGGAAACGATG-3'; Yong et al., 2006) and Vib2_R (5' - GAAATTCTACCCCCCTACAG-3'; Thompson et al., 2004; Vezzulli et al., 2012), resulting in a 511 bp product. In this instance, Mangomix™ (Bioline) Taq polymerase was used and the following cycling conditions were performed: an initial activation step at 95°C for 120 s, followed by 30 cycles of denaturation at 95° C for 15 s, annealing at 53° C for 30 s and extension at 72°C for 30 s, after which a final elongation step at 72° C for 10 min was performed. In both cases, PCR products were used to prepare DNA libraries with the Illumina TruSeq DNA library preparation protocol. Sequencing was performed, following an additional amplification step using the 27F-519R primer pair for the 16S rRNA amplicon sequences on an Illumina MiSeq (at Molecular Research LP; Shallowater, TX, USA) following the manufacturer's guidelines.

16S rRNA gene sequences were analyzed using the QIIME pipeline (Caporaso et al., 2010; Kuczynski et al., 2011). De novo Operational Taxonomic Units (OTUs) were defined at 97% sequence identity using UCLUST (Edgar, 2010) and taxonomy was assigned to the Greengenes database (version13_8; McDonald et al., 2012) using

BLAST (Altschul et al., 1990). Chimeric sequences were detected using ChimeraSlayer (Haas et al., 2011) and filtered from the dataset. Sequences were then rarefied to the same depth to remove the effect of sampling effort upon analysis (Santos et al., 2014) and chao1 diversity estimates were calculated. ANOVA was used (treatment) to determine significant differences ($P < 0.05$) between the diversity estimates in each treatment. Prior to this, data was tested for normality using the Kolmogorov–Smirnov test and Levene's test was used for homogeneity of variance. In cases where these assumptions were not met, $\log_{10}$ transformations were performed. The community composition for each of the treatments t_0, t_{final} Control, and t_{final} Heat Stress was averaged across the three replicates within each treatment.

Multivariate statistical software (PRIMER v6) was used to measure the degree of similarity between the bacterial commu-nity composition in each treatment (Clarke and Gorley, 2006). Data was square-root-transformed and the Bray–Curtis similarity was calculated between samples. Similarity percentage (SIMPER) analysis (Clarke, 1993) was used to identify the sequences con-tributing most to the dissimilarity between the treatments.

For the *Vibrio*-specific assay, the OTUs representing >1% of the total sequences were combined with various *Vibrio* species nucelotides taken from Yong et al. (2006) and *V. coralliilyticus* nucleotides taken from Huete-Stauffer et al. (unpublished), Ben-Haim et al. (2003b) and Ushijima et al. (2014) to build a phylogenetic tree. Sequences were first aligned and inspected using MUSCLE (Edgar, 2004) and the tree was constructed after 1,000 bootstrap re-samplings of the maximum-likelihood method using the Tamura-Nei model (Tamura et al., 2007) in MEGA 6.0 (Tamura et al., 2013), where only values >50% were displayed on the tree (Felsenstein, 1985). The OTU abundance was represented as a percentage of the overall community composition. OTUs were included on the tree if responsible for driving significant differences between the treatments according to SIMPER analysis and were color coded according to whether the OTU was more abundant in the t_{final} Control treatment (blue circle) or the t_{final} Heat stress treatment (red circle).

Quantitative PCR and Analysis

Quantitative PCR analyses targeting a *Vibri* specific region of the 16S rRNA gene and the heat shock protein gene (*dnaJ*) specific to *V. coralliilyticus* were conducted on all samples. Standards were created by growing the bacterial isolates *V. parahaemolyticus* (ATCC 17802) and *V. coralliilyticus* (ATCC BAA-450) overnight in Marine Broth (BD, Difco) at 37°C (150 rpm shaking water bath) and 28°C (170 rpm in a shaking incubator), respectively. Prior to qPCR analysis, calibration curves for each assay were created using viable counts from dilution series of the isolates. The cultures were homogenized and divided into 4 × 1 mL aliquots, washed three times with sterile phosphate-buffered saline (PBS) and pelleted at 5200 g for 10 min. Three of the washed pellets were used for DNA extraction using the MO BIO Ultra Clean Microbial DNA Kit (Carlsbad, CA, USA), while the remaining washed pellet was resuspended in 1 mL PBS and 10-fold serial dilutions with Phosphate Buffered Saline were prepared in triplicate. Three replicate 100 µL aliquots from each dilution (10^{-5}–10^{-8}) were spread onto marine agar plates and grown at 37°C (*V. parahaemolyticus*) or 28°C (*V. coralliilyticus*) over 24–48 h, and resultant colonies were counted.

A 1:5 dilution of DNA: nuclease free water was used for all qPCR assays to reduce pipetting errors. The *Vibrio* population was assessed using 16S rRNA *Vibrio* primers Vib1_F (5'-GGCGTAAAGCGCATGCAGGT-3') and Vib2_R (5'-GAAATTCTACCCCCCTACAG-3'; Thompson et al., 2004; Vezzulli et al., 2012) producing a 113 bp product. Power SYBR Select Master Mix (Applied Biosystems) was used, with reaction mixtures comprising 10 µL Master Mix, 5 µL of diluted (1:5) sample, and 0.4 µM of each primer to a final volume of 20 µL. The qPCR was performed using a Step One Plus (Applied Biosystems) and the following optimized cycling conditions: 2 min at 50°C, then an initial denaturation-hot start of 2 min at 95°C, followed by 40 cycles of the two-step reaction: 95°C for 15 s and 60°C for 1 min. This was followed by a holding stage at 72°C for 2 min and a melt curve stage.

The relative abundance of *V. coralliilyticus* was measured by targeting the *dnaJ* gene that encodes heat shock protein 40 in this species (Pollock et al., 2010), using the

primers: Vc_dnaJ_F1 (5'-CGGTTCGYGGTGTTTCAAAA-3') and Vc_dnaJ_R1 (5' - AACCTGACCATGACCGTGACA-3') and a TaqMan probe, Vc_dna-J_TMP (5'-6-FAM-CAGTGGCGCGAAG-MGBNFQ-3'; 6-FAM; Pollock et al., 2010). Reaction mixtures included a 10 µL TaqMan Universal Master Mix II (Applied Biosystems), 5 µL of diluted (1:5) sample, 0.6 µM of each primer and 0.2 µM fluorophore-labeled TaqMan probe in a final total volume of 20 µL. The optimized qPCR cycling conditions were: 2 min at 50°C, then an initial denaturation-hot start of 10 min at 95°C, followed by 40 cycles of the following incubation pattern: 95°C for 15 s and 60°C for 1 min. Resultant qPCR data for the *Vibrio*-specific and *V. coralliilyticus* assays were analyzed using Step One Software V2.3 (Applied Biosystems). The concentrations of bacteria were normalized to the coral surface area per cm^2, which was calculated by paraffin wax dipping as described in Holmes (2008) and Veal et al. (2010). To compare the abundance of bacteria in the t_0, t_{final} Control, and t_{final} Heat Stress treatments using the qPCR assays, ANOVA was used (treatment) to determine significant differences ($P < 0.05$) between the abundances in each treatment (qPCR). Prior to this, data was tested for normality using the Kolmogorov–Smirnov test and Levene's test was used for homogeneity of variance. In cases where these assumptions were not met, $\log_{10}$ transformations were performed.

Results

Effects of Elevated Temperature on Coral Health

No visual signs of stress or bleaching were evident in the Control nubbins over the course of the experiment, yet evidence of bleaching was observed in the Heat Stress nubbins where significant levels of heat stress of the zooxanthellae were detected in the t_{final} Heat Stress treatment compared to the t_{final} Control nubbins using PAM fluorometry. Heat stressed corals showed a strong decline in zooxanthellae condition (significant decrease in the F_V/F_M ($P = 0.002$) and Y[PSII] ($P = 0.003$) measurements; Supplementary Information Tables S4.1 and S4.2), while simultaneously the zooxanthellae were protecting their cells from further

photodamage by significantly increasing the xanthophyll cycle – Y[NPQ] measurements (Supplementary Tables S4.1 and S4.2).

Bacterial Community Composition

Differences in bacterial community composition between the t_{final} Control and t_{final} Heat Stressed corals were identified using 16S rRNA gene amplicon sequencing (Figure 4.1). The community composition of the t_{final} Control and t_{final} Heat Stress treatments were 42% dissimilar (SIMPER analysis; Supplementary Figure S4.1, Supplementary Table S4.3), while the largest difference (56%) in the community composition was between t_0 and the t_{final} Heat Stress treatments (Supplementary Information Table S4.4). Chao1 diversity estimates revealed that the t_{final} Heat Stress treatment had significantly ($P < 0.05$) higher diversity (1406 ± 155 SD) compared to the t_{final} Control (995 ± 23 SD).

The bacterial community at t_0 was dominated by the *Oceanospirillales* (47%), which were primarily composed of members from the *Endozoicomonacea,* followed by *Burkholderiales* (8.5%), *Rickettsiales* (7%), and *Rhodobacterales* (6%) (Figure 4.1). A shift in the community was observed in control corals over the 7 day experiment involving an increase in the relative occurrence of sequences matching the *Rhodobacterales* (21%) and *Flavobacteriales* (8.6%), and a decrease in *Oceanospirillales* sequences (29%). These shifts are indicative of a mild experimental effect (Figure 4.1). However, a dramatic community shift was detected in the t_{final} Heat Stress treatment relative to both the t_0 and t_{final} Control samples, which involved an increase in the relative proportion of *Rhodobacterales* (46.7%), *Flavobacteriales* (17.3%), and *Vibrionales* (10.5%). The occurrence of *Vibrionales* is notable because these organisms were not present in either control treatment (Figure 4.1). SIMPER analysis revealed that the decrease in *Oceanospirillales* abundance and increase in *Vibrionales* abundance were primarily responsible for differences in community composition between the t_{final} Control and Heat Stress treatments (Supplementary Table S4.3).

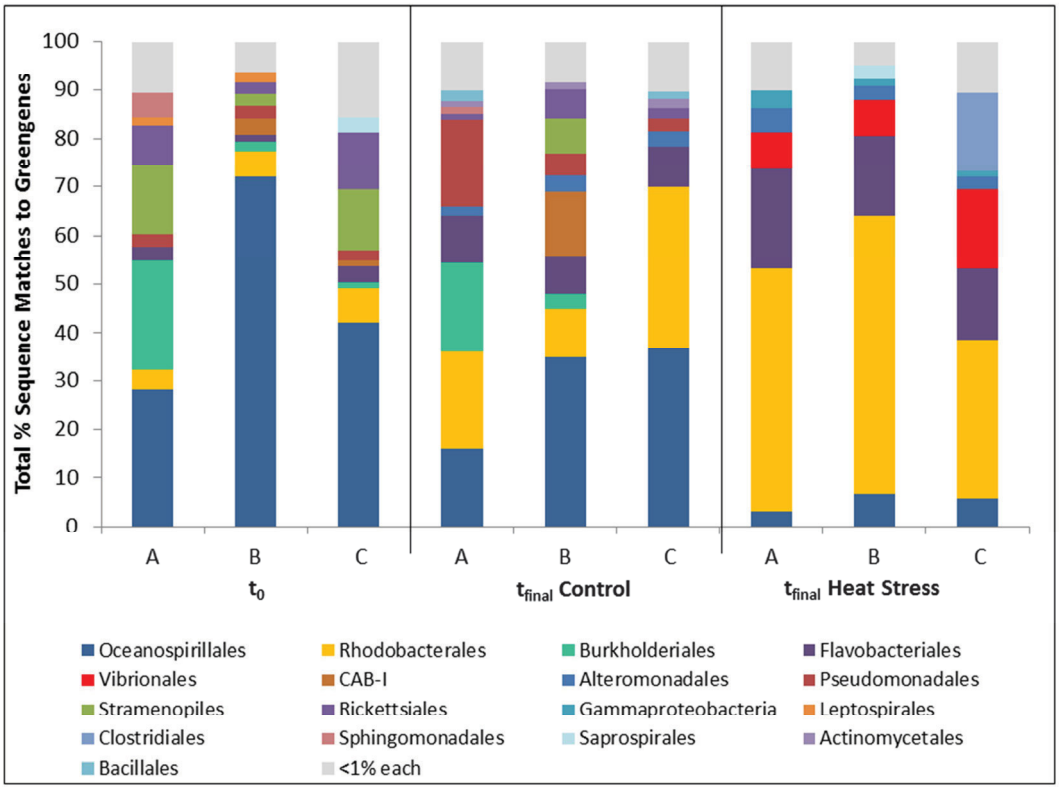


Figure 4.1 Bacterial taxa (order) associated with the coral *Pocillopora damicornis* on Heron Island, the Great Barrier Reef at t_0 (22°C), t_{final} Control (22°C) and t_{final} Heat stress (31°C) (A, B, C) conditions using 16S rRNA gene amplicon pyrosequencing. Hits were generated by comparing the sequences with BLASTn to the Greengenes database in QIIME

Quantification of the General *Vibrio* Population and of *V. coralliilyticus* Using Real Time qPCR

To confirm and quantify the increased abundance of *Vibrio* observed in t_{final} Heat Stressed corals, we applied a qPCR assay to track changes in the relative abundance of the *Vibrio* community. The *Vibrio* community-specific qPCR assay detected Vibrios in all treatments (Figure 4.2, standard curve: $R^2 = 0.99$, the t_{final} Heat Stress treatment, where they reached an average of 2.2×10^7 ($\pm 6.3 \times 10^6$ SD) cells cm^{-2} of coral surface ($P < 0.01$, Supplementary Table S4.5). *Vibrio* abundances in this treatment were two–three orders of magnitude higher than in the t_{final} Control [1.4×10^5 ($\pm 9.5 \times 10^4$ SD) cells cm^{-2}] and t_0 (2.0×10^4 ($\pm 1.5 \times 10^4$ SD) cells cm^{-2}) samples, respectively, (Figure 4.2). Variation in the abundance of the coral pathogen

V. coralliilyticus was also assessed using qPCR. In the t_0 corals, *V. coralliilyticus* was detected in only one of the three replicate colonies, in very low abundance (17.5 cells cm^{-2} of coral surface). Similarly, low concentrations were observed in the t_{final} Control samples, with abundances in one replicate below the detection limit and a mean of 81.5 cells cm^{-2} observed in the other two replicates. In contrast, *V. coralliilyticus* concentrations in the t_{final} Heat Stress treatment 6.3×10^4 ($\pm 3.4 \times 10^4$ SD) were significantly higher ($P < 0.05$, Supplementary Table S4.6) and reached up to four orders of magnitude higher than the t_{final} Control (Figure 4.3).

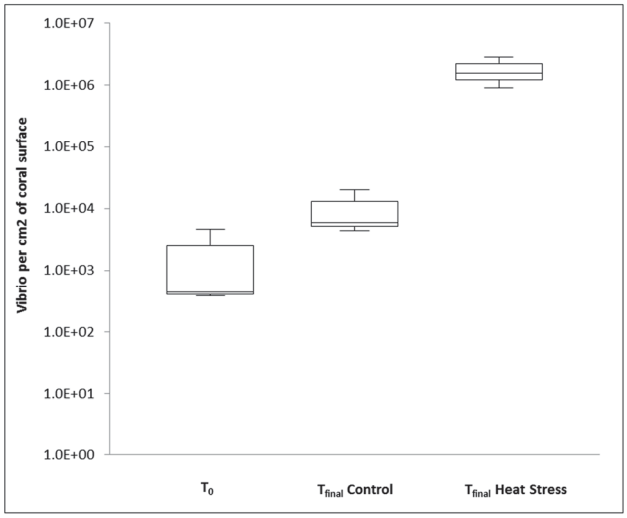


Figure 4.2 Real-time qPCR was performed to quantify the abundance of natural populations of vibrios associated with the coral *Pocillopora damicornis* on Heron Island, the Great Barrier Reef at t_0 (22°C), t_{final} Control (22°C) and t_{final} Heat stress (31°C) conditions. Standard curve: R^2=0.99, Eff%=93.1. Abundances are expressed as the number of bacteria per cm^2. n=3.

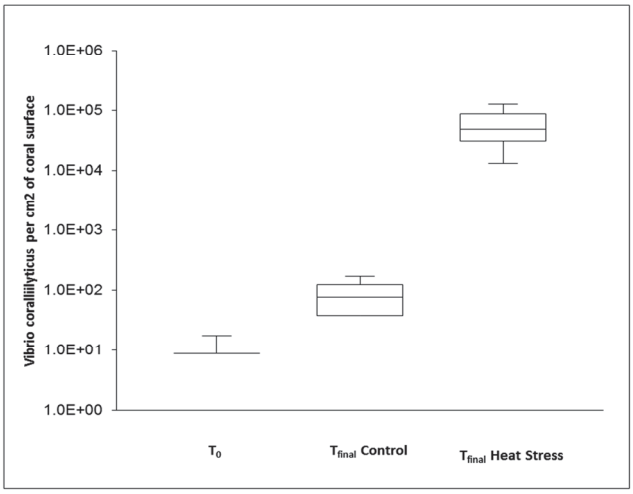


Figure 4.3 Real-time qPCR assays were used to quantify the abundance of natural populations of *Vibrio coralliilyticus* associated with the coral *Pocillopora damicornis* on Heron Island, the Great Barrier Reef at t_0 (22°C), t_{final} Control (22°C) and t_{final} Heat stress (31°C) conditions, standard curve: R^2=0.995, Eff%=99.9. Abundances are expressed as the number of bacteria per cm^2. n=3.

Characterising Changes in the *Vibrio* Population Induced by Heat Stress

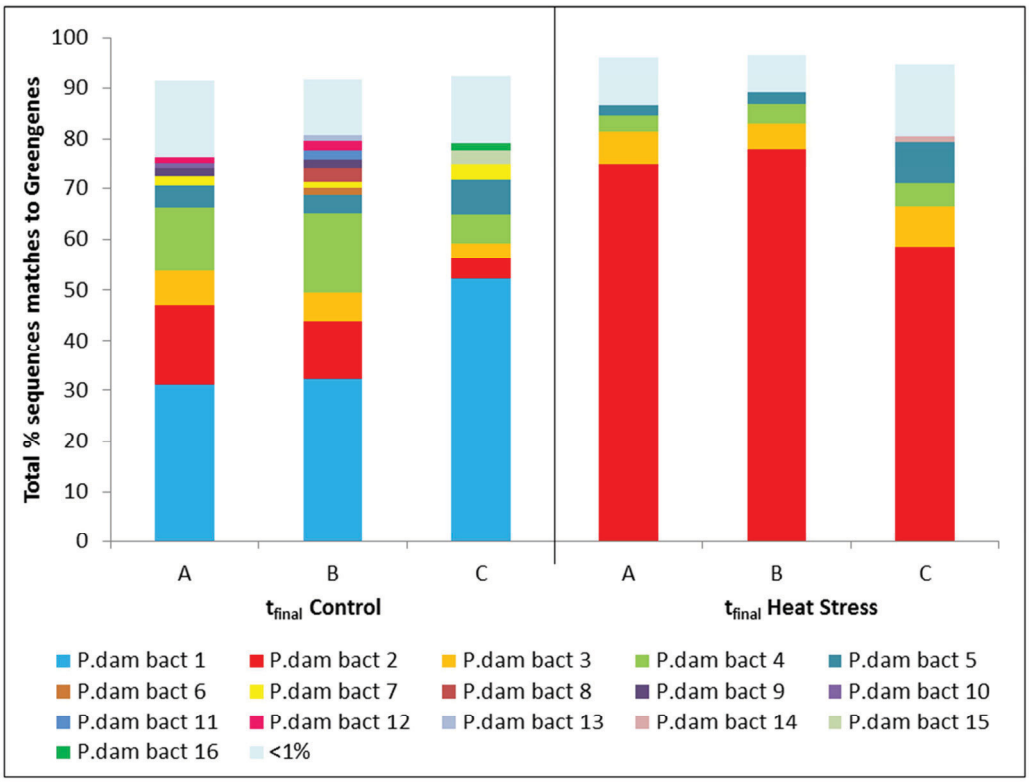


Fig. 4.4 Operational Taxonomic Units (OTUs) of the general *Vibrio* bacteria associated with the coral *Pocillopora damicornis* on Heron Island, the Great Barrier Reef at t_{final} Control (22°C) (A, B, C) and t_{final} Heat stress (31°C) (A, B, C) conditions.

Using a *Vibrio*-specific 16S rRNA amplicon sequencing approach we observed a clear shift in the composition of the coral *Vibrio* community between the t_{final} Control and t_{final} Heat Stress treatments. Consistent with the results of the qPCR assay, where negligible numbers of *Vibrio* were detected, a small number (n = 2024) of *Vibrio* sequences were observed in the t_0 treatment. To avoid rarefying to this very low number of sequences, the t_0 treatment was subsequently omitted from the data set, as we consider the key comparison to test for the effects of increased seawater temperatures to be the t_{final} Control vs. Heat Stress treatments. The *Vibrio* community composition was different between the t_{final} Control and Heat Stress treatments. In particular, two OTUs, denoted *P. dam* bact 1 and bact 2, were responsible for driving the largest differences (29 and 25%, respectively, according to SIMPER analysis)

between treatments (Figure 4.4, Supplementary Table S4.7). While the *P. dam* bact 1 OTU comprised an average of 38.5% (±6.8%) of the community in the t_{final} control treatment (Figure 4.4), it was not present in the t_{final} Heat Stress treatment. In contrast, the *P. dam* bact 2 OTU was more abundant in corals from the t_{final} Heat Stress treatment, comprising an average of 70.6% (±6.0%) of the total *Vibrio* community (Figure 4.4), while representing only 10.4% (±3.4%) of the community in the t_{final} control treatment. Phylogenetic analysis of the two dominant *Vibrio* OTUs (4.5) revealed that *P. dam* bact 1 appears to be closely related to *V. pomeryoi* (AJ491290), while *P. dam* bact 2 may be related to *V. tubiashii* (KJ094891.1) and *V. coralliilyticus* (KF864214.1; Figure 4.4).

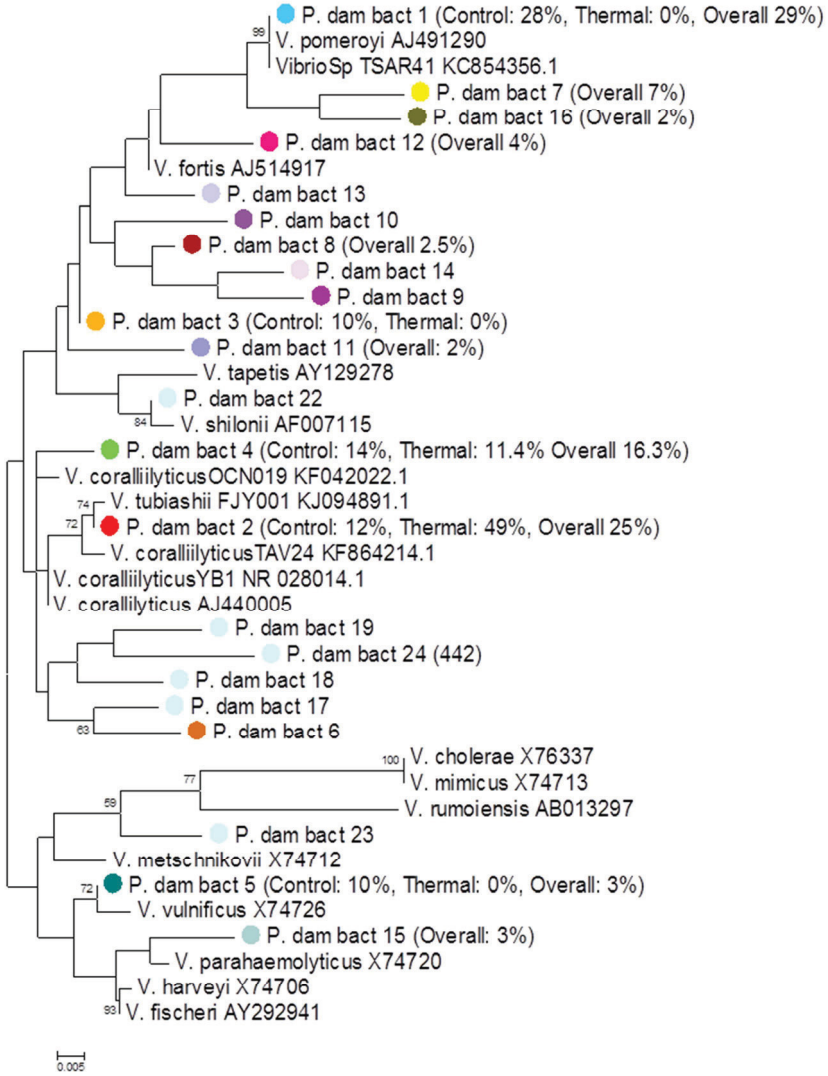


Fig. 4.5 Phylogenetic tree of the general *Vibrio* population. The colours of OTU circles match the colour of OTUs from Fig.4.4. The percentage abundances of the OTUs in the t_{final} Control and t_{final}

Heat Stress treatments are represented as a percentage of the total community composition only if the OTU is responsible for driving significant differences between the treatments according to SIMPER analysis (Supplementary table 4.6). The numbers at the nodes are percentages indicating the levels of bootstrap support, based on 1,000 resampled data sets where only bootstrap values of >50% are shown. The scale bar represents 0.005 substitutions per nucleotide position.

Discussion

Rising global temperatures, related to anthropogenically driven climate change, are expected to drive the geographical expansion of pathogens and the spread of disease outbreaks (Harvell et al., 1999, 2002; Burge et al., 2014). In marine habitats, a rise in *Vibrio*-induced diseases has been identified as an emerging global issue and has been correlated to rising seawater temperatures (Vezzulli et al., 2012; Baker-Austin et al., 2013). For instance, increasing seawater temperature has been linked to increased *Vibrio* occurrence in the North and Baltic Seas and a concurrent increase in cases of human infections by *Vibrio* species in this region (Vezzulli et al., 2012; Baker-Austin et al., 2013). Similarly, increasing numbers of human infections by *V. vulnificus* and *V. parahaemolyticus* off the coast of Spain have been linked to higher seawater temperatures (Martinez-Urtaza et al., 2010).

Clear links between elevated seawater temperature and the global decline of corals have also become increasingly apparent (Mydlarz et al., 2009; De'ath et al., 2012). Elevated seawater temperatures have led to (i) increased occurrence of coral bleaching, whereby symbiotic dinoflagellates are expelled from the coral host (Hoegh-Guldberg et al., 2007) and (ii) a situation where many corals are living close to their thermal physiological maximum (Hoegh-Guldberg et al., 2007). In addition to these direct effects on coral physiology and the coral-*Symbiodinium* symbiosis, rising seawater temperatures have also been linked to increased incidence of coral disease and microbial associated bleaching, or white syndrome (Bruno et al., 2007). In particular, *V. shiloi* and *V. coralliilyticus* have been identified as temperature-dependent pathogens responsible for coral bleaching (Kushmaro et al., 1996, 1997, 1998; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003a).

To date, the majority of research investigating the roles of *Vibrio* sp. in coral disease has been conducted in the laboratory using cultured isolates obtained from healthy and diseased corals (Kushmaro et al., 1998; Banin et al., 2000; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003a; Koren and Rosenberg, 2006; Vidal-Dupiol et al., 2011; Garren et al., 2014; Rubio-Portillo et al., 2014) with relatively few studies assessing natural populations of coral-associated *Vibrio* during heat stress or bleaching events (Bourne et al., 2008; Vezzulli et al., 2010). Community finger-printing approaches have previously revealed increases in the relative abundance of *Vibrio* populations during a naturally occurring bleaching event on the GBR (Bourne et al., 2008), while the appearance of *V. coralliilyticus* in diseased specimens of the octocoral *Paramuricea clavata* was also linked to elevated seawater temperature (Vezzulli et al., 2010).

In our study, initial evidence for a temperature induced increase in coral-associated *Vibrio* was provided by 16S rRNA gene amplicon sequencing. Corals from the control treatments were dominated by the *Oceanospirillales*, primarily due to the abundance of *Endozoicomonacea,* a group widely shown to be associated with healthy colonies of diverse coral species (Morrow et al., 2012, 2014; Bayer et al., 2013; Neave et al., 2014) including *P. damicornis* (Bourne and Munn*,* 2005). In contrast, the bacterial community in t_{final} Heat Stressed corals was characterized by significantly higher levels of diversity (Chao1) than the t_{final} Control corals. This is consistent with previous studies where diversity increased among white plague affected corals (Sunagawa et al., 2009). The t_{final} Heat Stressed corals contained diverse assemblages of copiotrophic and potentially opportunistic microbes including *Rhodobacteriales*, *Flavobacteriales,* and *Vibrionales*. Notably, while *Vibrio* sequences made up 10.5% of sequences in corals from the t_{final} Heat Stress treatment, they were completely absent in the t_0 and t_{final} Control samples. In addition, a substantial decrease in *Oceanospirillales* and a disappearance of *Burkholderiales* was observed in t_{final} Heat Stressed corals. The changes observed here are consistent with previous research indicating that specific bacterial populations, including putative pathogens, emerge, and dominate the coral-associated bacterial community during environmental stress events (Roder et al., 2014). These community shifts may be a

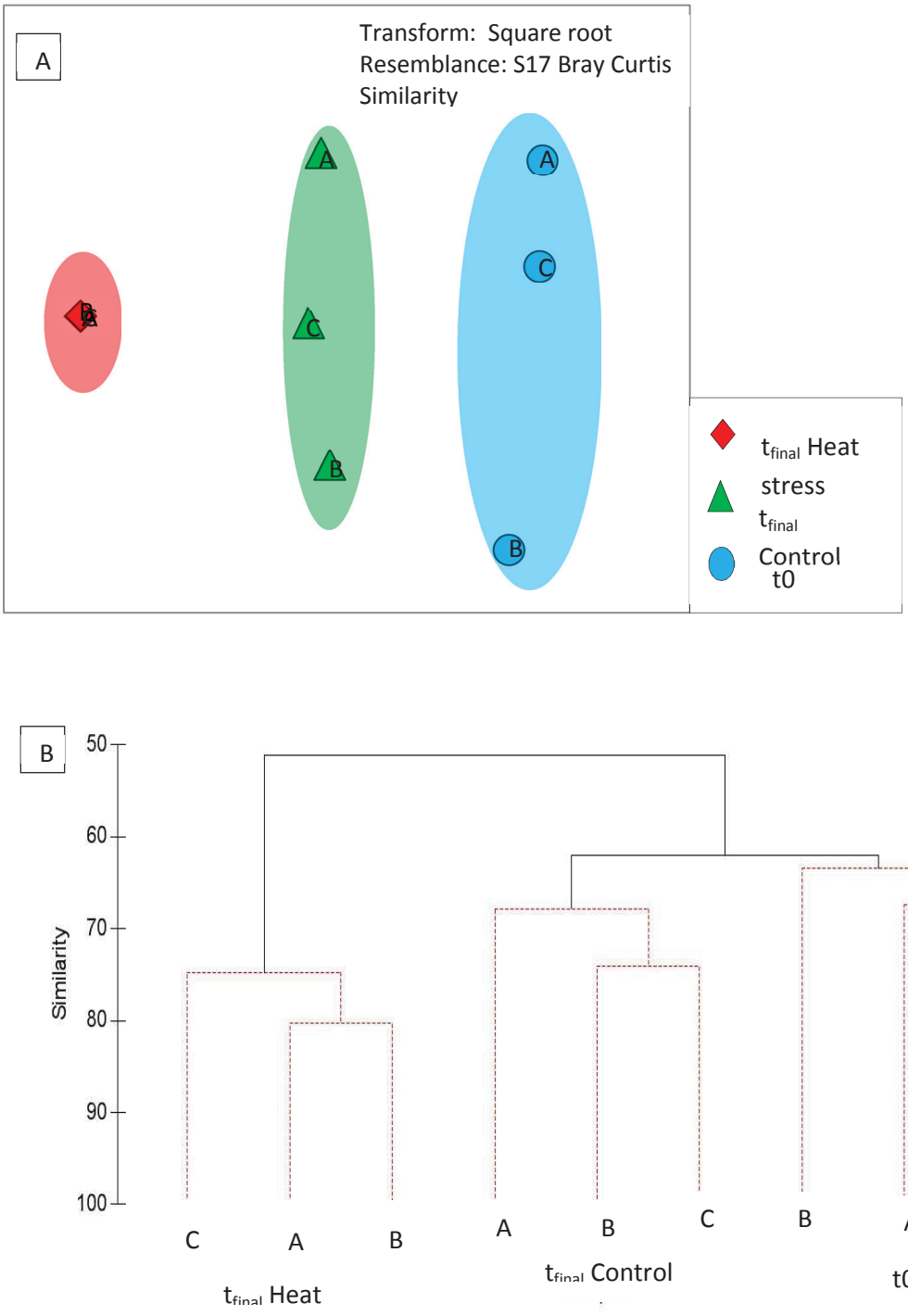
direct effect of temperature on the growth of specific members of the microbial community, or alternatively caused by a change in the chemicals released by heat-stressed corals (Garren et al., 2014). Due to the increased proportion of Vibrio sequences in the 16S rRNA amplicon analysis and the potential role of of *Vibrio* in coral disease (Vezzulli et al., 2010), we investigated the dynamics of this community further using targeted qPCR and Vibrio-specific amplicon sequencing approaches. A clear shift in the composition of the *Vibrio* community was observed in conjunction with the increased *Vibrio* abundance under elevated seawater temperature. Using qPCR, we detected low abundances of total *Vibrio* in the t_0 and t_{final} Control treatments, consistent with previous observations in healthy corals (Ritchie and Smith, 2004; Raina et al., 2009; Vezzulli et al., 2013) and our 16S rRNA amplicon sequencing results. However, we observed an increase in relative *Vibrio* abundance of two– three orders of magnitude in the t_{final} Heat Stressed corals. These patterns support previous reports that *Vibrio* abundance is linked to seawater temperature (Rubio-Portillo et al., 2014). While the increased abundance of *V. coralliilyticus* is part of a broader increase in abundance of total Vibrios, the magnitude of increase was substantially higher in *V. coralliilyticus* (four orders of magnitude compared to 2–3 orders of magnitude). This indicates that the putative coral pathogen *V. coralliilyticus* particularly benefited from the increased seawater temperature during in this study.

The ecological role of the resident *Vibrio* community in the health of corals is likely to vary substantially across species. Some Vibrios appear to form mutualistic relationships with corals by fixing nitrogen in the mucus (Chimetto et al., 2008) whereas others are putative agents of coral disease. However, despite substantial evidence of links between coral disease and *Vibrio* occurrence, in many cases it is unknown whether these organisms are the primary etiological agents or simply opportunistic colonizers that exploit the coral when host health is compromised (Bourne et al., 2008; Raina et al., 2010). While difficulties in assigning *Vibrio* taxonomy using 16S rRNA sequencing approaches are sometimes encountered (Cana-Gomez et al., 2011), our *Vibrio* specific 16S amplicon assay demonstrated clear differences between the *Vibrio* communities in the control and heat-stress samples and identified two key OTUs responsible for driving these differences. In the

control corals the *Vibrio* community was dominated by OTUs that matched *V. pomeroyi* (AJ491290), supporting previous research showing *V. pomeryoi* is found year round in healthy corals (Rubio-Portillo et al., 2014). *V. pomeryoi* is not known to be involved in coral disease and is likely a normal resident member of the coral-associated community (Rubio-Portillo et al., 2014). Up to 70% of the *Vibrio* community in t_{final} Heat Stressed corals was comprised of a single OTU (OTU *P. dam* bact 2), which our phylogenetic analysis indicates is closely related to the oyster pathogen *V. tubiashii* (KJ094891.1; Hada et al., 1984; Hasegawa et al., 2008; Richards et al., 2015) and the coral pathogen *V. coralliilyticus* (KF864214.1). *V. tubiashii* and *V. coralliilyticus* are highly related species (Ben-Haim et al., 2003b), and whilst the taxonomy of OTU *P. dam* bact 2 remains to be fully resolved, the phylogenetic positioning close to several *V. coralliilyticus* strains indicates that this organism may be *V. coralliilyticus*. This would be consistent with the findings of our *V. coralliilyticus* qPCR analysis, where a four orders of magnitude increase in abundance of *V. coralliilyticus* was observed in corals from the t_{final} Heat Stress treatment. These results are consistent with findings of Vezzulli et al. (2010) who only observed *V. coralliilyticus* in diseased coral specimens, as well as Ben-Haim and Rosenberg (2002) who, using cultured isolates of *V. coralliilyticus,* demonstrated that elevated temperatures are crucial to the infection of *P. damicornis*.

Our findings demonstrate, for the first time, that elevated sea water temperature increases the abundance and alters the composition of an environmental *Vibrio* community occurring among a mixed natural microbial community associated with the ecologically important coral species *P. damicornis*. Importantly, these microbial shifts involve a dramatic rise in the relative abundance of pathogens including *V. coralliilyticus*. Our research builds upon previous studies using cultured isolates, to highlight that natural populations of Vibrios, occurring within mixed natural communities of coral associated microbes may rise to prominence under heat stress conditions. Currently, up to a third of all coral species face extinction (Carpenter et al., 2008), with coral disease recognized as a significant and increasing threat. Our data provide direct quantitative support for the theory that increasing sea surface temperature occurring as a result of climate change, will affect coral reefs by promoting an increase in the abundance of coral pathogens.

Supplementary Information



Supplementary Figure S4.1 a) nMDS plot and **b)** CLUSTER analysis of the three treatments t_0, t_{final} Control and t_{final} Heat Stress.

Supplementary Table S4.1 Photosynthetic health of the corals were measured using PAM fluorometry was used at t_{final} Control and t_{final} Heat Stress treatments.

Measurement/ Treatment	Maximum quantum yield of PSII (F_V/F_M)	Effective quantum yield of Y(PSII)	Nonregulated nonphotochemical quenching (Y(NO))	Regulated non photochemical quenching (YNPQ)
t_{final} Control	64.8%	42.7%	23.6%	33.6%
t_{final} Heat Stress	51%	21.4%	28.9%	49.6%

Supplementary Table S4.2: A 1-way ANOVA was used to determine significant differences between the PAM fluorometry measurements at each treatment.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
F_VF_M	Between Groups	288.592	1	288.592	45.705	.002 ***
	Within Groups	25.257	4	6.314		
	Total	313.849	5			
Y(PSII)	Between Groups	680.328	1	680.328	40.757	.003 ***
	Within Groups	66.770	4	16.692		
	Total	747.098	5			
Y(NO)	Between Groups	41.910	1	41.910	.867	.405
	Within Groups	193.434	4	48.358		
	Total	235.344	5			
Y(NPQ)	Between Groups	384.521	1	384.521	18.271	.013 ***
	Within Groups	84.182	4	21.045		
	Total	468.703	5			

Supplementary Table S4.3: PRIMER table showing SIMPER analysis between the t_{final} Control and t_{final} Heat Stress treatments with an average dissimilarity of 41.78%.

Groups Thermal & control
Average dissimilarity = 41.78

Species	Thermal Av. Abund	Control Av. Abund	Av. Diss	Diss/SD	Contrib%	Cum.%
Oceanospirillales	0.22	0.53	3.92	2.79	9.39	9.39
Vibrionales	0.32	0.06	3.22	4.32	7.7	17.09
Rhodobacterales	0.68	0.45	2.98	1.66	7.14	24.23
Pseudomonadales	0.08	0.27	2.39	1.61	5.72	29.95
Burkholderiales	0.07	0.23	2.08	1.1	4.99	34.94
CAB-I	0.03	0.17	1.71	0.91	4.08	39.02
Clostridiales	0.14	0.02	1.59	0.71	3.8	42.82
Stramenopiles	0.02	0.15	1.58	1.36	3.79	46.61
Flavobacteriales	0.41	0.29	1.55	3.39	3.7	50.31
Rickettsiales	0.07	0.16	1.17	1.59	2.79	53.1
Actinomycetales	0.05	0.12	0.99	3.29	2.37	55.48

Supplementary Table S4.4: PRIMER table showing SIMPER analysis between the t_{final} Heat Stress and t_0 treatments with an average dissimilarity of 56.02%.

Groups Thermal & t0
Average dissimilarity = 56.02

Species	Thermal Av. Abund	t0 Av. Abund	Av. Diss	Diss/SD	Contri%	Cum.%
Oceanospirillales	0.22	0.68	5.73	2.5	10.23	10.23
Rhodobacterales	0.68	0.23	5.59	3.91	9.99	20.22
Vibrionales	0.32	0.03	3.5	4.46	6.24	26.46
Stramenopiles	0.02	0.29	3.32	2.93	5.92	32.39
Flavobacteriales	0.41	0.16	3.21	4.23	5.72	38.11
Rickettsiales	0.07	0.26	2.27	2.37	4.05	42.16
Burkholderiales	0.07	0.24	2.08	0.97	3.71	45.87
Clostridiales	0.14	0.02	1.56	0.7	2.79	48.67
Alteromonadales	0.18	0.06	1.51	3.17	2.69	51.35
Leptospirales	0	0.1	1.26	1.34	2.25	53.61
Gammaproteobacteria	0.14	0.04	1.2	2.57	2.14	55.74

Supplementary Table S4.5: A 1-way ANOVA was used to determine the significant differences between the abundances of Vibrios per cm^2 of *P. damicornis* for each treatment

One-way ANOVA: Abundance of Vibrio per cm^2 of P. damicornis V treatment

Source	DF	SS	MS	F	P
SAMPLE	2	9.76072E+14	4.88036E+14	12.40	0.007
Error	6	2.36120E+14	3.93533E+13		
Total	8	1.21219E+15			

S = 6273223 R-Sq = 80.52% R-Sq(adj) = 74.03%

Supplementary Table S4.6: A 1-way ANOVA was used to determine the significant differences between the abundances of *V. coralliilyticus* per cm^2 of *P. damicornis* for each treatment

SOURCE	DF	SS	MS	F	P
A: C1	2	102350	51174.98	10.25	0.011615*
S(A)	6	29965.56	4994.261		
Total (Adjusted)	8	132315.5			
Total	9				

* Term significant at alpha = 0.05

Supplementary Table S4.7: PRIMER table showing SIMPER analysis between the OTUs from the t_{final} Heat Stress and t_{final} Control treatments with an average dissimilarity of 46.43%.

CONTROL & THERMAL
Average dissimilarity = 46.43

	CONTROL	THERMAL				
Species	**Av.Abund**	**Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
P.dam bacteria OTU 1	6.16	0	13.75	5.57	29.62	29.62
P.dam bacteria OTU 2	3.13	8.39	11.77	4.05	25.35	54.97
P.dam bacteria OTU 7	1.43	0	3.21	3.48	6.91	61.87
P.dam bacteria OTU 4	3.28	1.98	2.85	1.9	6.13	68.01
P.dam bacteria OTU 9	0.91	0	1.95	1.33	4.21	72.21
P.dam bacteria OTU 12	0.8	0	1.72	1.32	3.71	75.92
P.dam bacteria OTU 5	2.21	1.94	1.64	2.3	3.52	79.44
P.dam bacteria OTU 15	0.56	0	1.31	0.67	2.82	82.26
<1%	3.6	3.2	1.24	1.47	2.66	84.92
P.dam bacteria OTU 8	0.55	0	1.15	0.67	2.48	87.4
P.dam bacteria OTU 11	0.45	0	0.94	0.67	2.03	89.43
P.dam bacteria OTU 16	0.4	0	0.93	0.67	2	91.43

Chapter 5:

Interrogating the effects of thermal stress on coral-microbe associations

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Abstract

The world-wide decline of coral reefs has been linked to the effects of global climate change. Here we examined how increased seawater temperature, expected as a consequence of climate change, influences the community composition and metabolic capability of natural populations of microorganisms associated with the coral *Pociliopora damicornis*. To replicate the 2-3°C increase in summer maxima predicted for 2100, seawater temperatures were increased to 31°C and 16S rRNA gene amplicon sequencing was combined with metagenomic and metatranscriptomic analyses to assess changes in the composition and function of the coral microbiome. Clear changes in bacterial community composition were evident at 31°C. Specifically, known coral-associates including members of the *Endozoicomonaceae* decreased dramatically (29% to 5%), while opportunistic and potentially pathogenic organisms such as the *Vibrionales,* which were absent at ambient temperature, increased in abundance to comprise up to 10% of the community in heat stressed corals. Metagenomic sequencing revealed shifts in the metabolic capability of the coral associated microbial community at elevated temperature including increased representation of clustering-based subsystems, membrane transport and respiration, as well as genes associated with virulence, disease and defence, stress responses and sulphur metabolism. Metatranscriptomic sequencing further revealed a significant upregulation of phage-associated genes and genes associated with protein metabolism, respiration, clustering-based subsystems and RNA metabolism. Notably, key behavioural functions including motility and chemotaxis, which have previously been shown to play a significant role in the virulence of coral pathogens, were up-regulated in the heat-stress metatranscriptome and were particularly highly expressed within *Vibrio* populations. Our results highlight how thermal stress causes significant changes in the composition and function of coral associated microbial communities including increases in the abundance of putative pathogens and up-regulation of key functions involved in the onset of coral disease.

Keywords: *Coral, Heat Stress, Pocillopora damicornis, Microbes, Metagenomics, Metatranscriptomics*

Introduction

Approximately 75% of the world's coral reefs are currently under threat from a range of local and global environmental pressures including overfishing, ocean acidification, catchment runoff and increasing seawater temperatures (Burke et al. 2011). In particular, rising seawater temperatures, linked to global climate change (Mouchka et al., 2010; Ruiz-Morenol et al., 2012), have multiple negative effects on the health of coral reefs and have been implicated as the cause of several large-scale coral die-offs (Bourne et al., 2009; Mydlarz et al., 2009; De'ath et al., 2012; Bourne and Webster, 2013). Repeated, severe and prolonged thermal stress events cause the symbiotic relationship between the coral host and the dinoflagellate *Symbiodinium* to uncouple, resulting in coral bleaching and high rates of mortality (Hoegh-Gulberg 2004a). Additionally, thermal tolerance or sensitivity varies among coral species, which may be the result of differing biochemical processes involved in temperature regulation including production of antioxidants, heat shock proteins and mycosporin-like amino acids (Brown et al 2002; Dunlap et al., 1986). Thermal stress can also directly influence the coral immune system, making corals more susceptible to pathogenic infections (Bourne et al., 2009; Mouchka et al., 2010). This is particularly relevant within the context of coral microbiology, because thermal stress can also cause shifts in the coral microbiome and is believed to increase the occurrence of pathogenic microbes responsible for microbial-driven bleaching, or white syndrome (Kushmaro et al., 1996, 1997, 1998; Ben-Haim and Rosenberg 2002; Ben-Haim et al., 2003).

Corals contain diverse and dynamic communities of microorganisms, including bacteria, archaea, fungi, protists, viruses and endolithic algae (Rosenberg et al. 2007). These microbial assemblages are typically more abundant and phylogenetically distinct from those in the surrounding seawater (Ducklow and Mitchell 1979a; Paul et al. 1986; Rohwer et al. 2001, 2002; Frias-Lopez et al. 2002; Kellogg 2004; Rosenberg et al. 2007; Sweet et al. 2011). Over large-scales, microbes play a fundamental role in the biogeochemical cycles of coral reefs (Sorokin 1978 a,b; Azam and Malfatti, 2007; Webster and Hill 2007; Falkowski et al., 2008), while at smaller scales bacteria drive important chemical processes within the coral

holobiont, including nitrogen fixation, which helps to sustain the relationship between the coral host and its symbiotic dinoflagellates (Lema et a., 2012). Microorganisms are also known to cause disease in corals, with microbially-mediated disease outbreaks now recognised as a key threat to coral reefs (Bourne et al., 2009; Burge et al., 2014). However, the effect and influence of coral-microbes is not static, rather these dynamic partnerships can display substantial spatiotemporal heterogeneity often linked to specific environmental drivers (Bourne et al., 2008), including changes in seawater temperature.

Temperature appears to be one of the major environmental regulators of coral-microbial interactions (Kushmaro et al. 1996, 1997 1998; Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003; Garren et al., 2014). As a consequence, understanding how the composition, behaviour and metabolic activity of microbial communities change in relation to thermal stress is vital to understanding and predicting the health of coral reefs (Bourne and Munn 2005; Roder et al. 2014). This is particularly relevant within the context of coral disease etiology and the ecology of potential coral pathogens.

Shifts in coral-associated bacterial assemblages directly influence the health of corals (Rhoder et al. 2014) and there is evidence that thermal stress influences i) the corals vulnerability to infection by pathogens (Hoegh-Guldberg, 1999; Hoegh-Guldberg and Hoegh-Guldberg, 2004; Jokiel and Brown, 2004); and ii) the virulence and behavioural characteristics of pathogenic bacteria (Vidal-Dupiol et al. 2011; Garren et al. 2014). Indeed, it has been demonstrated that elevated seawater temperature can lead to increases in virulence factors among specific bacterial pathogens, resulting in coral bleaching (Rosenberg and Ben-Haim 2002; Rosenberg et al. 2007). Elevated temperature has also been implicated in aspergillosis disease of sea fan corals in Florida (Ward et al., 2007) and black band disease in *Montipora* species on the Great Barrier Reef (Sato et al., 2009). Recently, it was shown that the coral pathogen *V. coralliilyticus* becomes increasingly chemotactic under elevated seawater temperatures, providing the pathogen with greater capacity to detect and colonize compromised coral hosts (Garren et al., 2014).

Shifts in the structure and function of coral-associated microbial communities under thermal stress have previously been reported to involve increases in the abundance of putative coral-pathogens and enhancement of their potential virulence (Vega-Thurber et al., 2009). However, while metagenomic studies such as these have revealed insights into the functional potential and virulence of coral microbial communities, metatranscriptomic approaches are better suited for assessing the influence of changing environmental conditions on functional variation and regulation within the transcriptionally active fraction of microbial communities at a particular time (Moran et al. 2013). Metatranscriptomics provides community-wide characterisation of gene transcripts (Stewart et al. 2010), without the need to pre-identify and target putative genes in the manner that is required with quantitative PCR (qPCR) or microarrays (Moran et al., 2009). Metatranscriptomic analysis has not been widely used to study coral microbiomes so elucidating the actively expressed microbial genes during thermal stress will generate important insights into how the coral holobiont responds to global change.

Materials and Methods

Heat stress experiment

Please refer to chapter 4 of this thesis (Tout et al., 2015b) for details on the heat stress experiments.

Photosynthetic health of corals

Please refer to chapter 4 of this thesis (Tout et al., 2015b) for details.

Coral-bacterial cell separation

Please refer to chapter 4 of this thesis (Tout et al., 2015b) for details and to Supplementary figure S5.1 for details concerning experimental design.

Genomic DNA concentrations were measured using a Qubit 2.0 fluorometer (Invitrogen) and ranged between 7.82 ng/µL – 48.7 ng/µL.

RNA isolation and extraction

The three treatments for each donor colony were used for RNA extractions, resulting in nine samples (Supplementary Figure S5.1). RNA extractions were performed on the pellet retrieved from the coral-bacterial cell separation process using the RiboPure Bacteria Kit (Ambion, Life Technologies, Carlsbad, CA, USA). Turbo DNase was used in substitution for the DNase I treatment to remove genomic DNA (Stewart et al., 2010). After DNase I treatment, RNA was purified and concentrated using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA). For each treatment, triplicate colony samples were pooled to obtain sufficient RNA. RNA concentration and quality was monitored during each step using a Qubit 2.0 fluorometer (Invitrogen) and a Nanodrop spectrophotometer (Thermo Scientific) respectively. Total RNA concentrations ranged between 12.2-14.5 ng/µL.

Microbial mRNA Enrichment and Isolation, rRNA Subtraction and Eukaryotic mRNA Removal

Bacterial RNA was isolated using the MICROBEnrich kit (Ambion) to remove any remaining host or eukaryotic RNA. Briefly, 160 ng of total RNA was used per sample and we assumed the samples contained 100% eukaryotic RNA for calculating the amount of Capture Oligo Mix and Oligo MagBeads. The MICROBExpress (Ambion) protocol was used to further enrich and purify bacterial mRNA which was isolated using the MICROBEnrich (Ambion) protocol.

Amplification of Enriched mRNA, cDNA Synthesis and Sequencing

Following eukaryotic RNA removal, bacterial RNA isolation and mRNA enrichment, 109 ng of enriched bacterial mRNA per sample was amplified using the MessageAMP II Bacteria kit (Ambion) (Frias-Lopez et al. 2008; Shi et al. 2009) according to the methods of Stewart et al. (2010), whereby the T7-*Bpm*I-$(dT)_{16}$VN

primer was used for reverse transcription to convert to double stranded cDNA. cDNA was purified and transcribed for 14 hours at 37 °C *in-vitro* to produce a total of 1-13 µg of single-stranded anti-sense RNA per sample. 500 ng of aRNA from each sample was purified and converted to double stranded cDNA using the SuperScript III First-Strand synthesis System kit (Invitrogen, Carlsbad, CA, USA) using random-hexamer primers (Stewart et al. 2010). Second-strand cDNA synthesis was performed with the SuperScript Double Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA) (Stewart et al. 2010) beginning at the second-strand synthesis step and finishing after step 3 when cDNA was purified with a PCR clean up kit (Mo BIO, Carlsbad, CA, USA). Finally, poly(A) tails were removed with a *BpmI* digestion stage for 3 hours at 37 °C, followed by a heat deactivation step at 70 °C for 20 minutes. Poly(A)-removed cDNA was purified using a PCR clean Up kit (Mo BIO, Carlsbad, CA, USA) resulting in a total of 18-256 ng of cDNA. From this cDNA, 40 µL aliquots were stored at -80 °C until use for generating libraries with the Nextera-XT kit (Illumina).

16S rRNA Amplicon Sequencing and Analysis

Please refer to chapter 4 of this thesis (Tout et al., 2014b) for details and supplementary figure S5.1.

Metagenomic and Metatranscriptomic Analysis

9 Metagenomic and 3 Metatranscriptomic libraries were generated using the Nextera-XT Kit (Illumina) and sequencing was performed on the Illumina HiSeq2000 platform (Australian Centre for Ecogenomics, University of Queensland, Australia) according to the Nextera-XT Kit protocol. Sequences were subsequently analysed using the *Meta Genome Rapid Annotation using Subsystems Technology* (MG-RAST; Version 3 pipeline (Overbeek et al., 2005; Meyer et al., 2008)). Quality control was performed by removing reads with >10 ambiguous bases per read and de-replicating artificial duplicates in which the first 15 bp of the read were identical (Table 5.1). Within MG-RAST, metabolic assignments were annotated to the SEED subsystems database (Overbeek et al., 2005) and taxonomic identification was

determined based on the top BLAST hits to the SEED taxonomy. Matches with an E-value of <0.05 were considered significant with a minimum alignment of 15 bp (Vega-Thurber et al., 2009; Jeffries et al., 2012; Edwards et al., 2010; Pfister et al., 2010; Poroyko et al., 2010; Delmont et al., 2011; Jeffries et al., 2011; Seymour et al., 2012; Smith et al., 2012). All data was normalised to sequencing effort by dividing by the total number of hits per sample. Taxonomy was assigned to specific metabolic categories, which were over-represented in the metatranscriptomes using the work-bench feature in MGRAST.

Metabolic reconstructions generated using MG-RAST were imported into the Statistical Analysis of Metagenomic Profiles (STAMP) package to test for statistically significant differences between the 9 metagenomes and 3 metatranscriptomes. Fisher's exact test was used to determine significant differences between samples with a Benjamini FDR multiple test correction applied (Benjamini and Hochberg, 95; Rivals et al., 2007; Parks and Beiko, 2010). Hence, all quoted q-values represent corrected values (equating to P), with only values <0.05 reported (Parks and Beiko, 2010). Confidence intervals (95%) were determined using the Newcombe–Wilson method. Multivariate statistical software (PRIMER v6) was used to measure the degree of similarity between treatment types for all sequencing analyses (Clarke and Gourley, 2006). Data was square-root transformed and the Bray-Curtis similarity was calculated between samples. SIMPER analysis (Clarke, 1993) was used to identify the phylogenetic groups and metabolic categories contributing most to the dissimilarity between the metagenomes.

Results

Effects of elevated temperature on coral health

Please refer to chapter 4 of this thesis (Tout et al., 2014b) for details.

Bacterial community composition

Please refer to chapter 4 of this thesis (Tout et al., 2014b) for details. nMDS analysis showed that the bacterial communities differed according to treatment (Fig. 5.1a), where microbial community composition of corals in the control and heat stress treatments were 42% dissimilar (SIMPER analysis) (Supplementary Table 5.1,

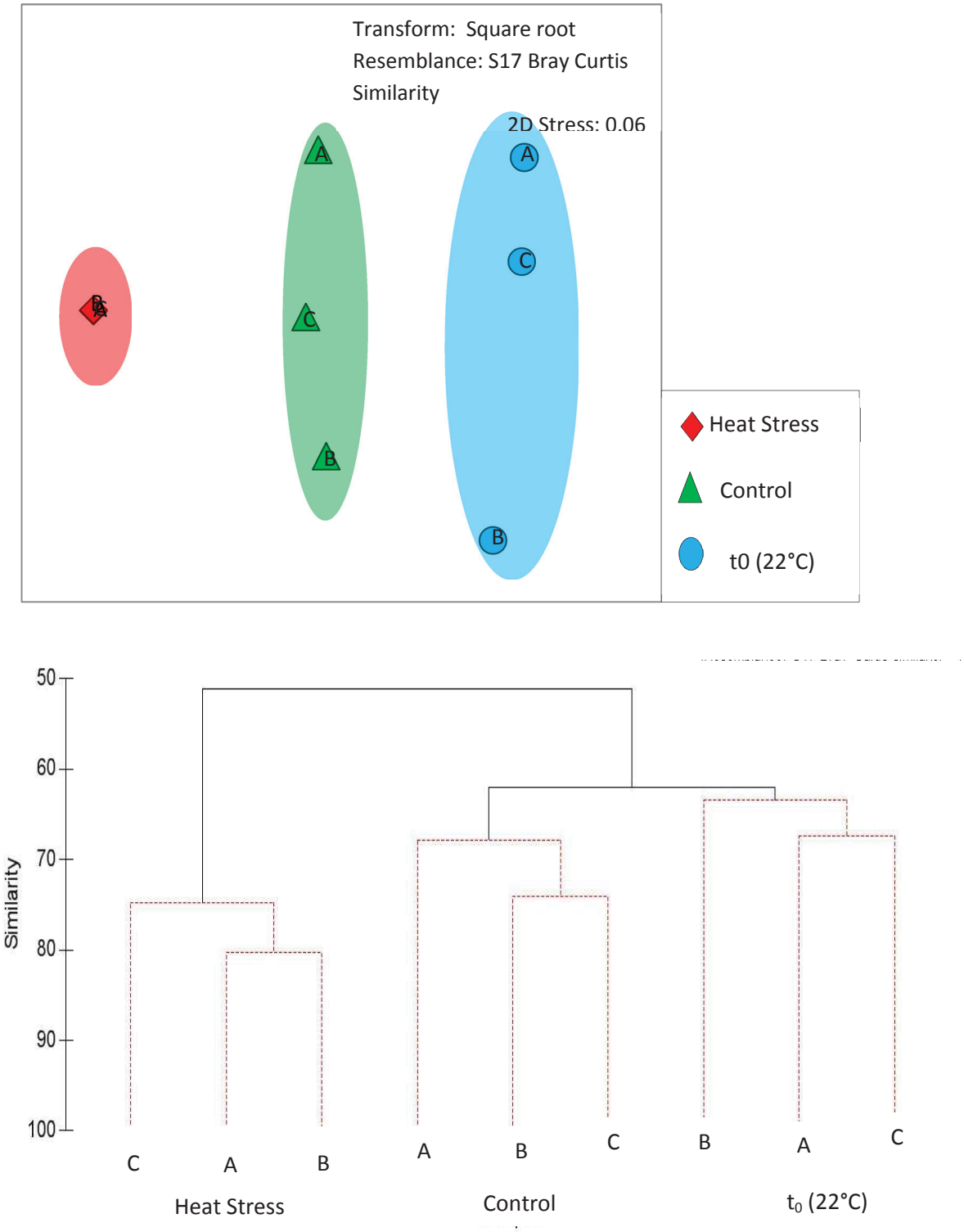


Figure 5.1 nMDSplot (a) and cluster analysis (b) of the 16S rRNA communities from the different treatments.

Fig. 5.2), while the largest difference (56%) in the community composition was between t_0 and the heat stress treatments (Supplementary Table 5.2).

Metagenomic characterisation of shifts in community functional potential

The metagenomes comprised between 1,134,737 to 1,590,536 sequences, with read lengths of 165 - 166 bp (Table 5.1). Comparisons with the SEED database revealed significant changes in the metabolic potential of the microbial communities between the different treatments using Fisher's exact test. nMDS analysis revealed that the t_0 and control treatments grouped together, while the heat stress replicates grouped separately (Fig. 5.2). SIMPER analysis revealed the heat stress and control metagenomes shared an average dissimilarity of 8.44% (Supplementary Table 5.4). Sequence matches to genes fundamental to core microbial functions, often referred to as "house-keeping" genes comprised the most abundant hits (up to 14.7%) across all metagenomes. These functions included DNA metabolism, clustering-based subsystems, carbohydrate metabolism, protein metabolism, amino acid metabolism, respiration and phage-associated genes (Fig. 5.3). Of these high abundance metabolic categories, Fisher's exact test ($q<0.05$) revealed that 12 SEED metabolic categories were significantly different between the control and heat stress treatments, with 37.8% of these being over-represented in the control metagenomes and 62.2% being

Table 5.1: Metagenome and Metatranscriptome statistics for the three treatments

	Metagenomes (average)			**Metatranscriptomes**		
Meta data	**t0**	**Control**	**Heat stress**	**t0**	**Control**	**Heat stress**
DNA sequences	1,225,762	1,134,737	1,590,536	7,735,488	9,402,072	15,079,443
Sequences fail QC	129,886	116,238	180,214	6,899,649	8,463,673	13,708,622
Mean sequence length of DNA base pairs (post-QC)	166	165	166	152	153	147
Known annotated proteins %	14	14	15	4.7	4.4	4.3
Eukaryote vs Bacterial composition %	56.2 v 43	54.53 v 44.5	49.8 v 49.3	N/A	N/A	N/A

over-represented in the heat stress metagenomes. SIMPER analysis revealed that the under-representation of phage-associated genes in the heat stress metagenomes was responsible for the greatest significant difference between metagenomes (15.1%) (Supplementary Table 5.3). Several other metabolic categories drove differences between the two treatments, including membrane transport and respiration, as well as genes associated with virulence, disease and defence, stress response and sulphur metabolism, which were all significantly more abundant in the heat stress metagenome (Supplementary Table 5.3).

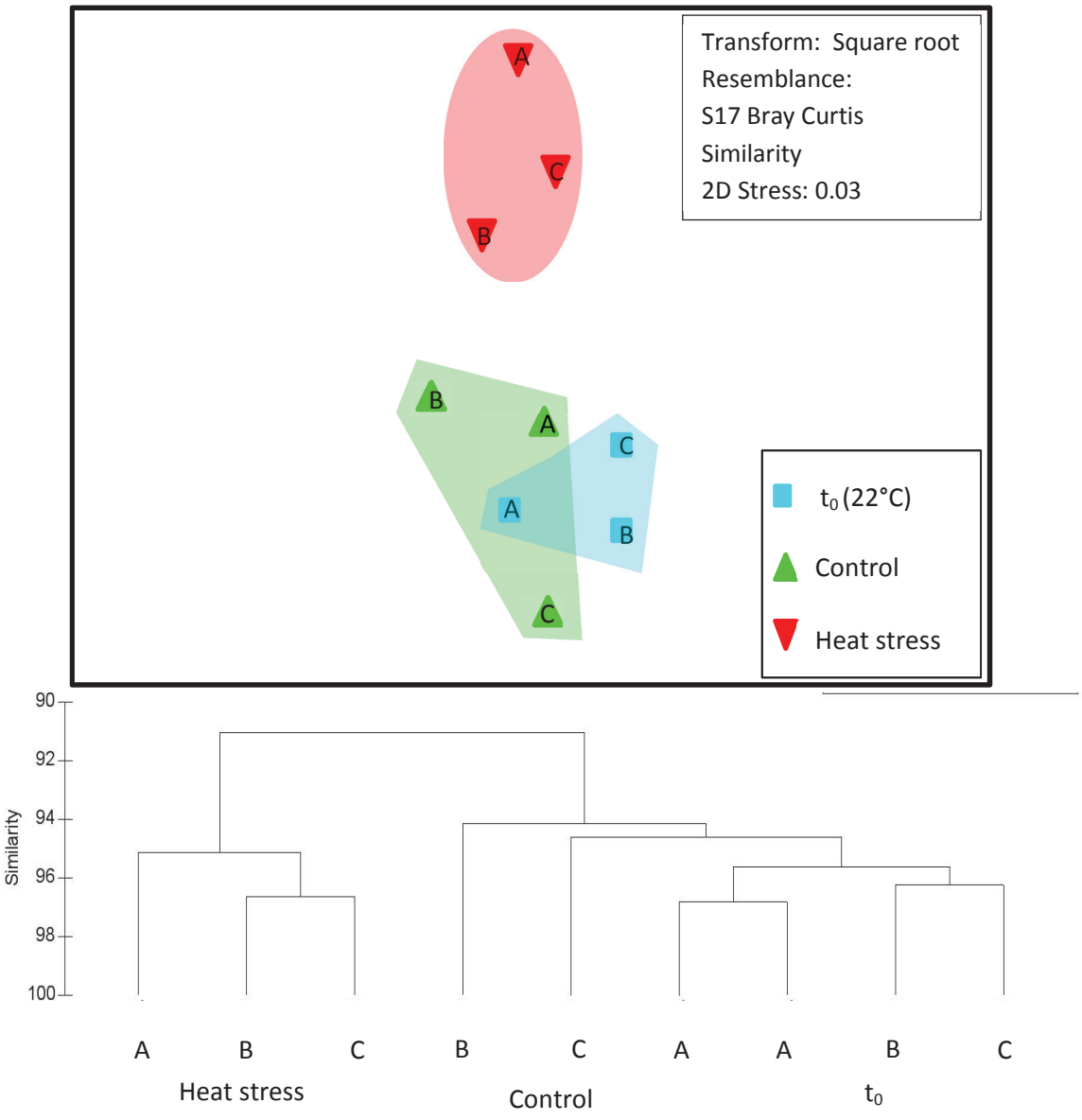


Figure 5.2 nMDS plot (a) and cluster analysis (b) of the functional categories (SEED Lvl 1) from the three metagenomes (t_0, control and heat stress; replicates A, B and C).

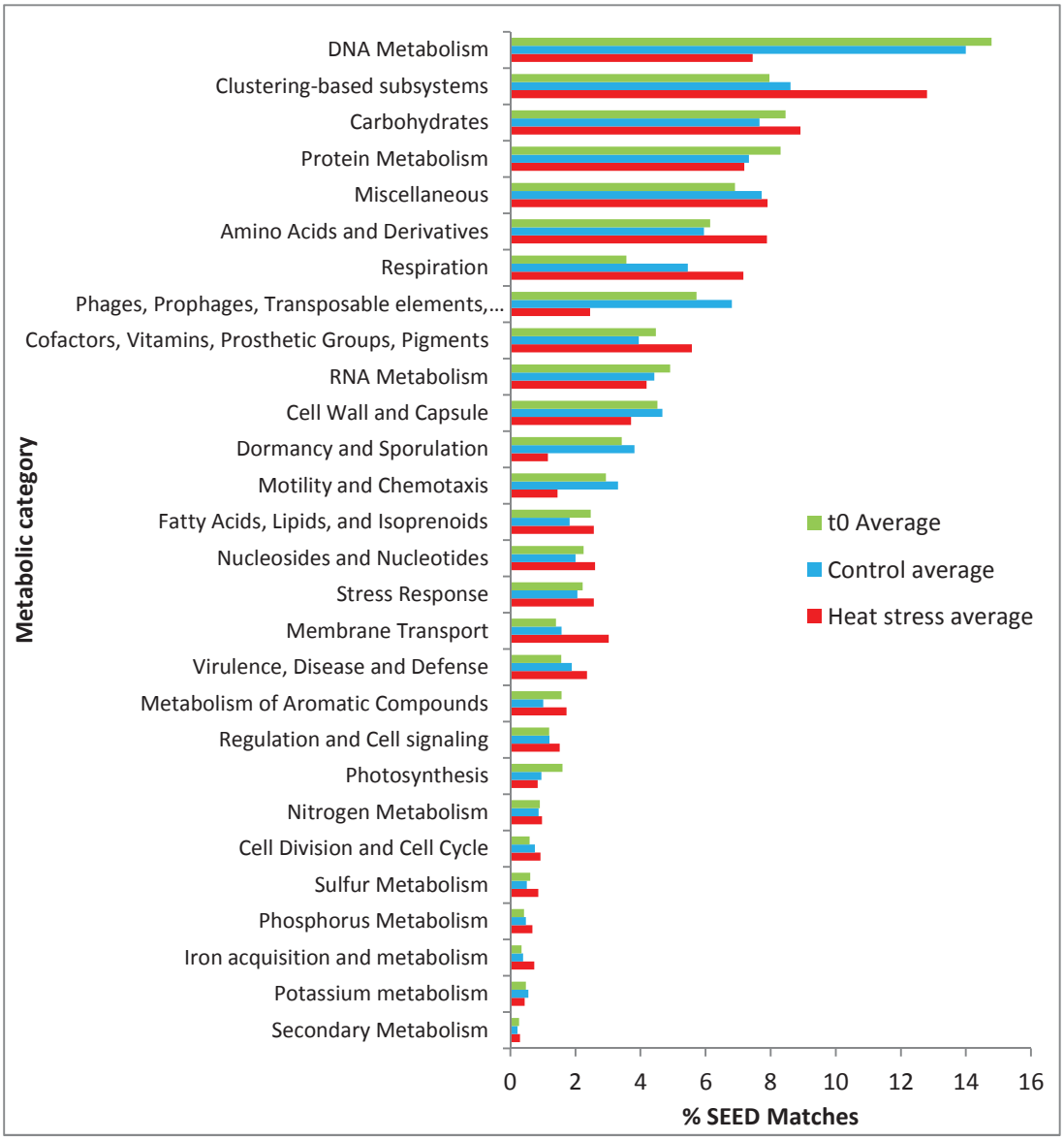


Figure 5.3 Frequency distribution (relative to percentage of microbial SEED matches) of microbial metabolic functions in each treatment from the metagenomes: t_0, control and heat stress (Ordered occurring to frequency in each treatment).

Metatranscriptomics

The metatranscriptomes were comprised of between 7,735,488 to 15,079,443 sequences, with read lengths of 147 to 153 bp (Table 5.1). Consistent with the metagenomes, sequence matches to "house-keeping" genes including respiration, clustering-based subsystems, protein metabolism, carbohydrate metabolism, amino acid metabolism and photosynthesis comprised the most abundant hits across all metatranscriptomes (Fig. 5.4). However, significant shifts in the genes being actively

expressed in the microbial communities across the different treatments were also observed. SIMPER analysis revealed that the control and the heat stress metatranscriptomes had an overall dissimilarity of 7.95% (Supplementary Table 5.4). Fisher's exact test revealed that 26 SEED categories were significantly different between the control and heat stress treatment (Fig. 5.5), of these, 65.4% were over-represented in the control while the remaining 34.6% were over-represented in the heat stress treatments. Genes associated with protein metabolism, phage-associated genes, respiration, clustering-based subsystems, RNA metabolism, and motility and chemotaxis were significantly ($q<0.05$) over-represented in the heat stress metatrancriptome (Fig. 5.5-5.6). Of these categories, the largest significant differences between the two treatments were driven by protein metabolism (11.8%) and phage associated genes (9.6%) (Supplementary Table 5.4).

Genes associated with respiration were significantly more abundant in the heat stress metatranscriptomes and the main taxonomic matches were to *Alteromonas macleodii, Flavobacterium psychrophilum*, *Polaribacter sp.* MED152 and several *Vibrio* species including *V. parahaemolyticus*, *V. harveyi, V. vulnificus* and *V. splendidus* (Fig. 5.7). The organisms that up-regulated motility and chemotaxis genes in the heat stress metatranscriptome included a variety of *Vibrio* species, including *V. vulnificus*, *V. harveyi*, *V. parahaemolyticus*, *V. splendidus* and *V. cholera* (Fig. 5.7).

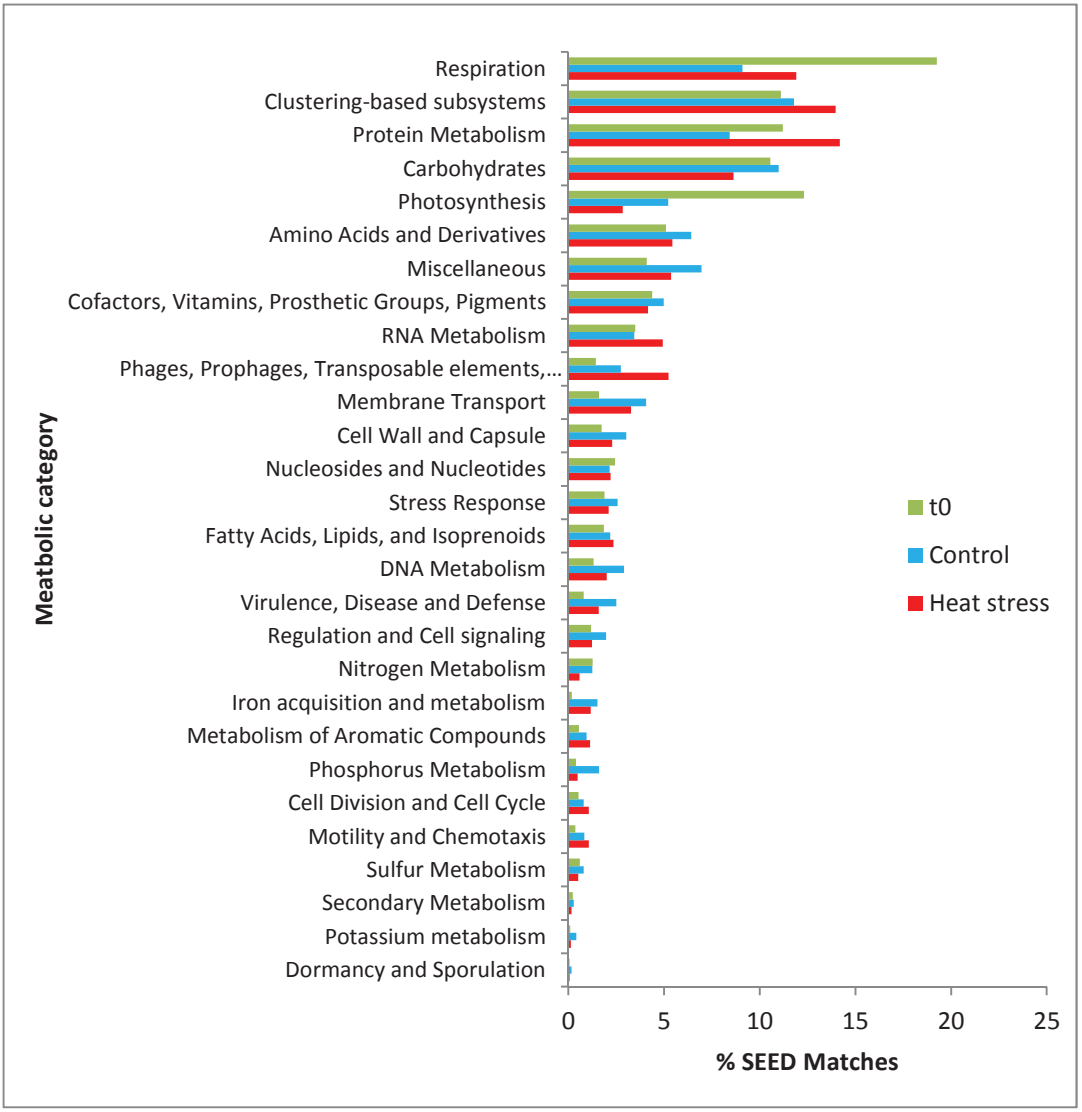


Figure 5.4 Frequency distribution (relative to percentage of microbial SEED matches) of microbial metabolic functions in each treatment from the metatranscriptomes: t_0, control and heat stress (Ordered occurring to frequency in each treatment).

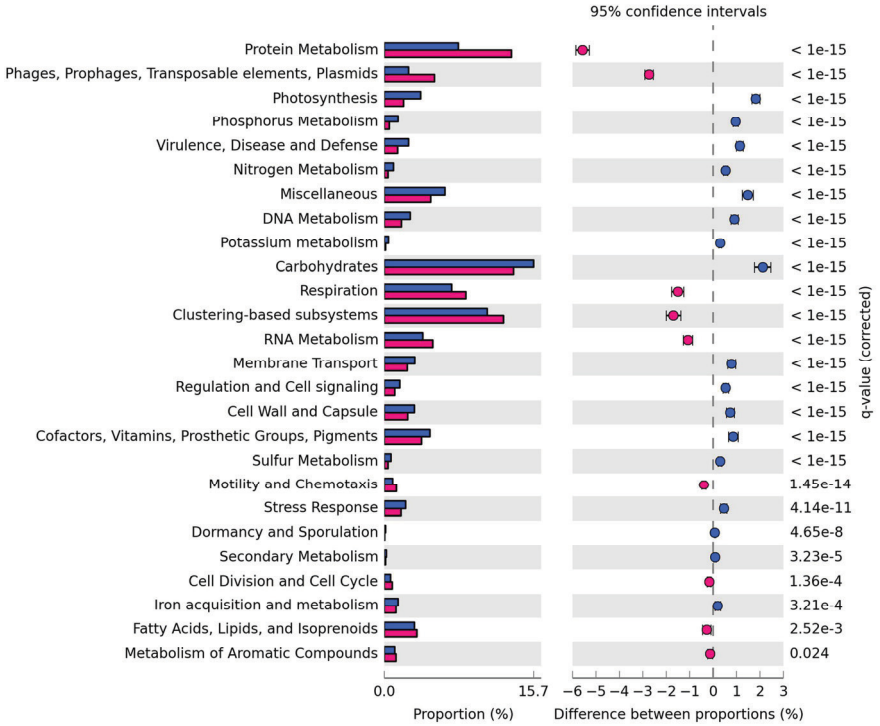


Figure 5.5 Fisher's exact test was used to statistically test for significant differences in the relative abundance of functional categories (SEED database) where functional groups over-represented in the heat stress metatranscriptome (red) correspond to negative differences between proportions while functional groups over-represented in the control metatranscriptome (blue) correspond to positive differences between proportions.

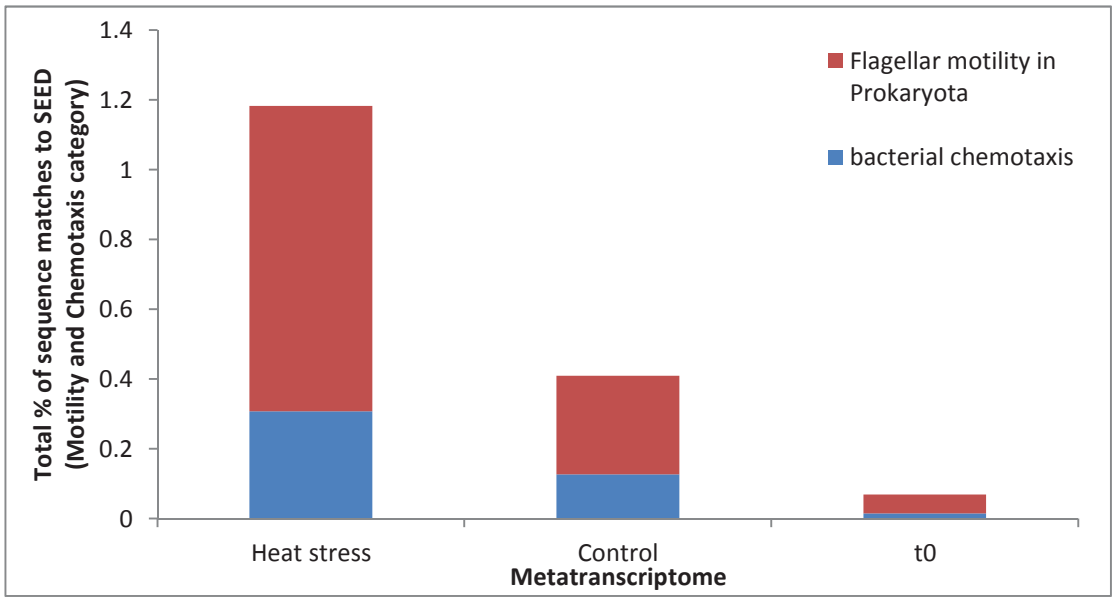


Figure 5.6 Functional categories of motility and chemotaxis composition of three metatranscriptomes derived from three treatments by comparing the sequences with BLASTn to the SEED database. Relative representation in the metatranscriptome was calculated by dividing the number of hits to each category by the total number of hits to all categories, thus normalising by sequencing effort.

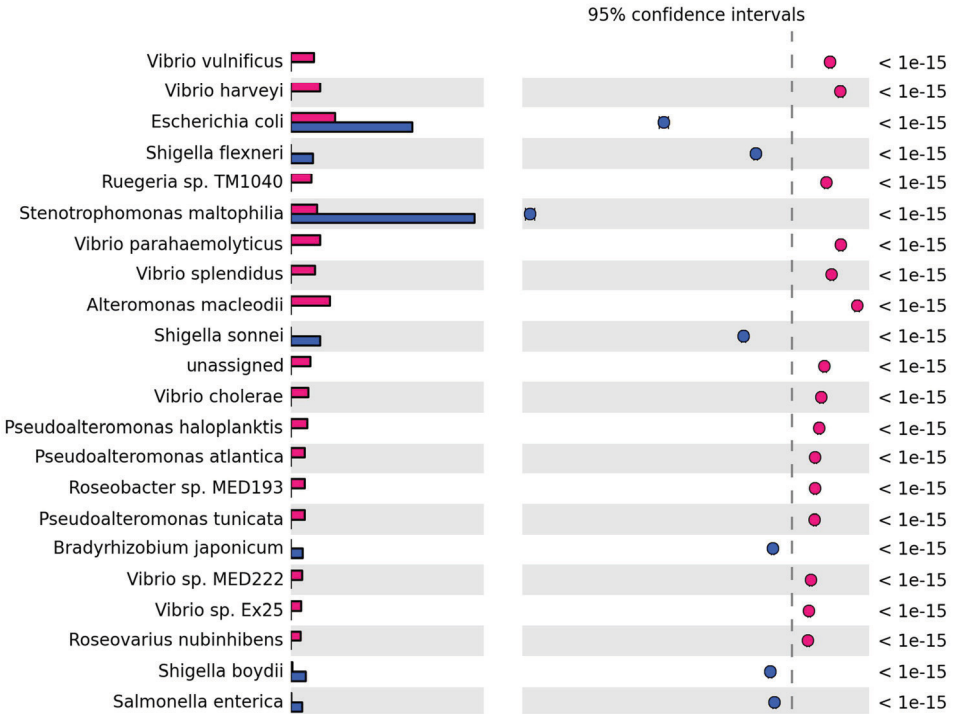


Figure 5.7 Fisher's exact test was used to statistically test for significant differences in the relative abundance of taxa (species level) matching bacterial functional chemotaxis and motility genes in the SEED database where species over-represented in the control metatranscriptome (blue) correspond to negative differences between proportions while species over-represented in the heat stress metatranscriptome (red) correspond to positive differences between proportions. Note: only the top 22 taxa are included in this figure for clarity reasons.

Discussion

Although not widely applied within the context of coral reef ecosystems, metagenomics has provided some important advances in our understanding of the functional capabilities of complex and diverse coral reef-associated bacterial communities (Fan et al., 2013; Tout et al., 2014). However, revealing which genes are being actively expressed is the crucial next step to understand how coral-associated microbial function varies under different environmental conditions. While the functional responses of the coral host and its zooxanthellae to elevated seawater temperatures have previously been examined using transcriptomic approaches (Vidal-Dupiol et al., 2011 Gust et al., 2014; Mayfield et al., 2014; Shinzato et al., 2014), it remains unknown how the regulation of gene expression in the coral-

microbial community changes in response to increasing seawater temperatures. This is particularly pertinent given that increasing seawater temperatures have been linked to increased coral disease and microbe-related bleaching (Bruno et al., 2007). To gain a better understanding of the functional responses of coral associated microbial communities to heat stress events, we employed metatranscriptomics, metagenomics and 16S rRNA amplicon sequencing to track changes in microbial community composition, potential function and active gene expression.

Marked changes in the coral-associated bacterial community occurred in response to increasing seawater temperature. *Oceanospirillales* dominated the coral community in the control treatment, which was primarily driven by the occurrence of the *Endozoicomonacea* family, which are a well-known core component of the bacterial assemblage associated with *P. damicornis* (Bourne et al. 2005) and many other coral species (Morrow et al., 2012; Bayer et al. 2014; Morrow et al., 2014; Neave et al. 2014). In line with our findings where *Oceanospirillales* decreased under heat stress, it has previously been demonstrated that coral stress caused by elevated CO_2 concentrations led to a decrease in the abundance of coral-associated *Endozoicomonacea* (Morrow et al., 2014). There is indeed evidence that following environmental stress events, core microbial community members decrease in abundance and specific bacterial populations including putative pathogens, emerge to dominate the coral-associated bacterial community (Bourne et al., 2008; Roder et al., 2014). Our results support this pattern, revealing that increased seawater temperature induces a shift in the composition of the coral associated bacterial community to one reflective of a copiotrophic and potentially pathogenic community. For instance, significant increases in the relative number of *Vibrio, Rhodobacterales* and *Flavobacteriales* sequences were observed in heat stressed corals. Members comprising the *Rhodobacterales* including Rhodobacter spp. have been shown to be present among diseased corals throughout the globe (Mouchka et al., 2010), suggesting they could be key members of a consortia of microbes which are always present in diseased corals. While more recently *Rhodobacterales* presence has been noted among the diseased Florida Keys coral *Montastraea faveolata* (Morrow et al., 2012).

The increased representation of Vibrio observed in heat stressed corals is significant as Vibrios are known to significantly alter the functional potential of the coral-associated microbial community during times of thermal stress (Vega-Thurber et al., 2009), shifting it to a more pathogenic or virulent state. Vibrios are also dominant members of the microbial communities associated with other benthic invertebrates during periods of temperature stress (Fan et al., 2013). Despite this, the majority of coral reef Vibrio research has been conducted in the laboratory using cultured isolates (Kushmaro et al., 1998; Toren et al., 1998; Banin et al., 2001; Ben-Haim and Rosenberg, 2002; Ben-Haim et al., 2003b; Garren et al., 2014), thus our understanding of coral-Vibrio dynamics within natural mixed microbial populations is limited. Mechanisms behind the temperature-induced shift in the community composition observed here may include i) increased growth and activity of copiotrophic microbes, including the *Vibrios*, *Rhodobacterales* and *Flavobacteriales*, driven by shifts or increases in the chemicals released by the coral (Raina et al., 2013), or ii) bacterial behavioural responses towards chemical cues emitted by a thermally stressed and compromised host coral (Garren et al., 2014). These observations illustrate that increased seawater temperature influences the composition of coral associated bacterial communities, which may in some instances lead to a potentially pathogenic bacterial community. However, the next important step is to understand how the functional capacity of these microbial communities changes in response to heat stress.

To date, only a handful of studies have used metagenomics to profile both the taxonomy and function of coral reef microbiomes and the majority of these have demonstrated shifts in microbial metabolic potential and taxonomic community structure across entire reef ecosystems as well as within single reefs (Wegley et al., 2007; Dinsdale et al., 2008; Bruce et al., 2011; Littman et al., 2011; Tout et al., 2014). Additionally, metagenomics has been used to track changes in the microbial community associated with coral-reef invertebrates under environmental stress. For instance, metagenomic analysis of *Acropora millepora* during a naturally occurring bleaching event on the Great Barrier Reef revealed an increase in virulence genes associated with bleached corals and a shift in metabolism from autotrophy to heterotrophy (Littman et al., 2011). Metagenomic analysis also demonstrated that

increasing seawater temperature causes a dramatic change in the metabolic capacity of the microbial community associated with *Porites compressa*, where genes associated with sulphur and nitrogen metabolism, stress resistance, virulence fatty acid and lipid utilisation, secondary metabolisms and motility and chemotaxis become over-represented (Vega-Thurber et al., 2009). In the coral reef sponge *Rhopaloeides odorabile,* metagenomics analysis revealed that increasing seawater temperature leads to the loss of sponge-symbionts that are typically associated with healthy sponges, and an increase in rapid-growing, opportunistic copiotrophs (Fan et al., 2013). Consistent with the present study, Fan and colleagues found that the functional potential of the resident sponge-microbes under ambient conditions was dominated by house-keeping genes including cell signalling, regulation and stress responses. However, genes associated with flagellar biosynthesis and EAL domain proteins increased in the sponge-microbe community under elevated temperatures, indicating a potential functional shift from house-keeping and symbiosis to motility and colonisation (Fan et al., 2013).

To uncover patterns of gene expression in response to thermal stress, we coupled metagenomics with a metatranscriptomic sequencing approach. In line with previous marine (Venter et al., 2004; Bourne et al., 2005; Rusch et al., 2007) and coral reef (Wegley et al., 2007; Dinsdale et al., 2008; Vega-Thurber et al., 2009; Littman et al., 2011; Bruce et al., 2012; Fan et al., 2013; Tout et al. 2014) 'omic' studies, genes associated with core "house-keeping" functions, such as protein, amino acid and carbohydrate metabolism, respiration and photosynthesis dominated both the metagenomes and metatranscriptomes. However, increased expression of protein metabolism, respiration and RNA metabolism in the microbial communities of heat stressed corals was responsible for driving some of the largest significant differences between treatments which is reflective of increased metabolic rates in marine heterotrophic bacteria during elevated temperatures (Pomeroy and Wiebe 2001).

The functional categories responsible for driving the largest significant difference between the control and heat stress metagenomes were genes relating to phages, prophages, transposable elements and plasmids. The decreased abundance of phage-associated functional genes in the metagenomes from heat stressed corals may signal

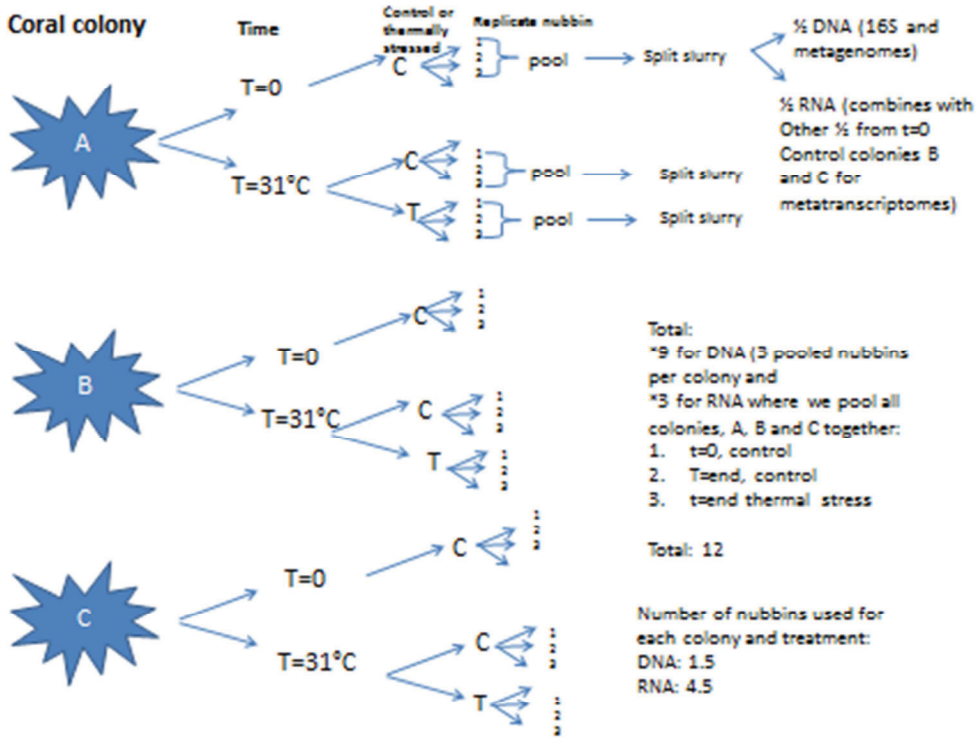
a decrease in the holobiont's defence against resident or opportunistic pathogens (Soffer et al., 2014) as bacteriophage can regulate the health of corals by controlling microbial over growth via predation (Wegley et al., 2007; Soffer et al., 2014). Consistent with the reduced abundance of phage-associated genes in our heat stressed corals exhibiting signs of bleaching, bacteriophage have also been shown to be less abundant in bleached corals (Soffer et al., 2014). A previous coral transcriptomic study highlighted that the coral *P. damicornis* responds to pathogen infection following heat stress by up-regulating genes involved in immune response, including pathogen recognition and antibacterial response (Vidal-Dupiol et al., 2011).

In this study, motility and chemotaxis was the only non-core functional category where active gene expression was significantly higher in the thermal stress metatranscriptome than in the control. This is particularly significant given the importance of chemotaxis in maintaining coral-bacteria associations (Garren et al. 2014, Tout et al. 2015) and the potential onset of disease in compromised corals (Garren et al. 2014). An increase in motility and chemotaxis genes affiliated with *Vibrio* has previously been shown in the coral *P. astreoides* in response to elevated seawater temperatures (Vega-Thurber et al., 2009) and results from our current study also indicate that natural populations of *Vibrio* are largely responsible for the up-regulation of motility and chemotaxis when seawater temperatures rise. Previous studies using cultured *Vibrios*, including the coral pathogens *Vibrio shiloi* (Kushmaro et al. 1996, 1997, 1998) and *V. coralliilyticus* (Ben-Haim and Rosenberg 2002; Ben-Haim et al. 2003), have demonstrated increased motility and chemotactic capacity under elevated seawater temperatures, and that these behavioural shifts are essential for the pathogens to locate and infect corals (Banin et al 2001; Meron et al. 2009; Vega-Thurber et al. 2009). Furthermore, *V. coralliilyticus* has been shown to up-regulate the expression of motility and chemotaxis genes under elevated seawater temperatures (Kimes et al., 2011) and displays extremely fast chemotactic responses towards the mucus of thermally stressed *P. damicornis* (Garren et al., 2014). Genes associated with motility and chemotaxis are abundant among natural communities of coral associated bacteria (Tout et al. 2015a) and have recently been reported as drivers of functional differences across microbial communities inhabiting different

coral reef microniches (Tout et al., 2014). In addition, flagellar-associated genes were significantly over-represented in the metagenomes of sponge-microbial communities during elevated sea water temperatures (Fan et al., 2013). Furthermore, using both laboratory and *in-situ* experiments we demonstrated high levels of chemotactic capacity among natural communities of coral associated bacteria, including *Vibrios* (Tout et al. 2015a). Together with these previous findings, our results confirm the ecological importance of chemotaxis and motility in coral microbiomes and demonstrate that elevated seawater temperature can lead to important functional changes in the holobiont.

Increasing seawater temperature dramatically alters the microbial composition in *P. damicornis* inducing a shift away from a community dominated by well-known coral-associates such as the *Endozoicomonacea,* to a community dominated by the *Rhodobacterales* and the putatively pathogenic *Vibrio*. Increasing seawater temperature also affects the genes being actively expressed by the microbial community including an increase in respiration reflective of increased metabolic rates as well as an increase in more specialised functions previously shown to be important in coral-microbes interactions, such as motility and chemotaxis. Notably, the enhanced expression of motility and chemotaxis was driven by up-regulation within potentially pathogenic *Vibrio* species that concurrently increase in abundance within heat stressed corals. These findings demonstrate that rising seawater temperatures associated with global climate change may fundamentally impact coral-microbial interactions and alter the microbial functions occurring within the coral holobiont.

Supplementary Information



Supplementary figure S5.1: Diagram representing experimental design including different *Pocillopora damicornis* coral colonies (A, B and C), time (t=0 and t=31°C=end), treatment (control or thermally stressed), replicate nubbins for each colony, time and treatment (1, 2 and 3) which were then divided between DNA (16S and metagenomes*) and RNA (metatranscriptomes*) extractions, (*denotes pooling of DNA or RNA).

Supplementary Table 5.1: PRIMER table showing SIMPER analysis between the Control and Heat Stress treatments with an average dissimilarity of 41.78%.

Groups Thermal & control
Average dissimilarity = 41.78

Species	Thermal Av. Abund	Control Av. Abund	Av. Diss	Diss/SD	Contrib%	Cum.%
Oceanospirillales	0.22	0.53	3.92	2.79	9.39	9.39
Vibrionales	0.32	0.06	3.22	4.32	7.7	17.09
Rhodobacterales	0.68	0.45	2.98	1.66	7.14	24.23
Pseudomonadales	0.08	0.27	2.39	1.61	5.72	29.95
Burkholderiales	0.07	0.23	2.08	1.1	4.99	34.94
CAB-I	0.03	0.17	1.71	0.91	4.08	39.02
Clostridiales	0.14	0.02	1.59	0.71	3.8	42.82
Stramenopiles	0.02	0.15	1.58	1.36	3.79	46.61
Flavobacteriales	0.41	0.29	1.55	3.39	3.7	50.31
Rickettsiales	0.07	0.16	1.17	1.59	2.79	53.1
Actinomycetales	0.05	0.12	0.99	3.29	2.37	55.48

Supplementary Table 5.2: PRIMER table showing SIMPER analysis between the t_{final} Heat Stress and t_0 treatments with an average dissimilarity of 56.02%.

Groups Thermal & t0
Average dissimilarity = 56.02

	Thermal	t0				
Species	Av. Abund	Av. Abund	Av. Diss	Diss/SD	Contri %	Cum.%
Oceanospirillales	0.22	0.68	5.73	2.5	10.23	10.23
Rhodobacterales	0.68	0.23	5.59	3.91	9.99	20.22
Vibrionales	0.32	0.03	3.5	4.46	6.24	26.46
Stramenopiles	0.02	0.29	3.32	2.93	5.92	32.39
Flavobacteriales	0.41	0.16	3.21	4.23	5.72	38.11
Rickettsiales	0.07	0.26	2.27	2.37	4.05	42.16
Burkholderiales	0.07	0.24	2.08	0.97	3.71	45.87
Clostridiales	0.14	0.02	1.56	0.7	2.79	48.67
Alteromonadales	0.18	0.06	1.51	3.17	2.69	51.35
Leptospirales	0	0.1	1.26	1.34	2.25	53.61
Gammaproteobacteria	0.14	0.04	1.2	2.57	2.14	55.74

Supplementary Table 5.3 PRIMER table showing SIMPER analysis between the thermal stress and control metagenomes with an average dissimilarity of 8.44%

Groups Control & Thermal stress

Average dissimilarity = 8.44

Species	Group Control Average abundance	Group Thermal stress Average abundance	Average Dissimilarity	Dissimilarity /SD	Contribution %	Cumulative %
Phages, Prophages, Transposable elements, Plasmids	2.76	1.56	1.28	3.84	15.1	15.1
DNA Metabolism	3.27	2.4	0.92	2.03	10.86	25.97
Dormancy and Sporulation	1.64	0.89	0.81	1.67	9.57	35.54
Clustering-based subsystems	2.79	3.42	0.67	3.14	7.92	43.45
Membrane Transport	1.08	1.53	0.48	2.01	5.67	49.12
Motility and Chemotaxis	1.58	1.15	0.47	2.87	5.52	54.64
Respiration	2.01	2.31	0.39	1.23	4.67	59.31
Cofactors, Vitamins, Prosthetic Groups, Pigments	2.02	2.35	0.35	2.67	4.18	63.5
Metabolism of Aromatic Compounds	1.04	1.32	0.29	1.68	3.49	66.98
Amino Acids and Derivatives	2.95	3.22	0.29	2.22	3.43	70.41
Iron acquisition and metabolism	0.74	0.93	0.28	1.47	3.32	73.73
Photosynthesis	0.82	0.75	0.26	1.19	3.03	76.76
Virulence, Disease and Defense	1.24	1.44	0.22	1.6	2.62	79.37
Stress Response	1.35	1.5	0.17	1.41	1.97	81.34
Phosphorus Metabolism	0.73	0.88	0.16	1.56	1.91	83.25
Cell Division and Cell Cycle	0.77	0.89	0.16	1.29	1.86	85.11
Carbohydrates	3.38	3.52	0.15	1.96	1.8	86.91
Fatty Acids, Lipids, and Isoprenoids	1.88	2.01	0.14	1.95	1.69	88.6
Sulfur Metabolism	0.71	0.83	0.12	1.69	1.47	90.07

Supplementary Table 5.4 PRIMER table showing SIMPER analysis between the thermal stress and control metatranscriptomes with an average dissimilarity of 7.95%

Groups Thermal stress & Control

Average dissimilarity = 7.95

Species	Group Thermal stress Average abundance	Group Control Average abundance	Average dissimilarity	Diss/SD	Contribution %	Cumulative%
Protein Metabolism	3.66	2.79	0.93	Undefined!	11.75	11.75
Phages, Prophages, Transposable elements, Plasmids	2.29	1.59	0.76	Undefined!	9.57	21.32
Photosynthesis	1.42	1.96	0.59	Undefined!	7.36	28.69
Phosphorus Metabolism	0.71	1.21	0.54	Undefined!	6.77	35.46
Virulence, Disease and Defense	1.19	1.6	0.44	Undefined!	5.59	41.06
Nitrogen Metabolism	0.64	0.97	0.35	Undefined!	4.46	45.52
Miscellaneous	2.21	2.53	0.34	Undefined!	4.26	49.78
DNA Metabolism	1.35	1.65	0.33	Undefined!	4.16	53.93
Potassium metabolism	0.35	0.66	0.33	Undefined!	4.1	58.03
Carbohydrates	3.69	3.96	0.3	Undefined!	3.77	61.8
Respiration	2.93	2.66	0.29	Undefined!	3.67	65.46
RNA Metabolism	2.26	2.01	0.27	Undefined!	3.43	68.9
Clustering-based subsystems	3.54	3.29	0.27	Undefined!	3.37	72.26
Membrane Transport	1.55	1.79	0.26	Undefined!	3.22	75.49
Regulation and Cell signalling	1.04	1.27	0.25	Undefined!	3.14	78.63
Cell Wall and Capsule	1.57	1.79	0.24	Undefined!	2.99	81.62
Cofactors, Vitamins, Prosthetic Groups, Pigments	1.98	2.19	0.22	Undefined!	2.81	84.42
Sulfur Metabolism	0.64	0.84	0.22	Undefined!	2.71	87.13
Motility and Chemotaxis	1.12	0.93	0.21	Undefined!	2.63	89.76
Stress Response	1.33	1.5	0.17	Undefined!	2.19	91.96

Summary & Discussion

The research presented in this thesis has been inspired by a need to explore the ecology, function and behaviour of naturally occurring communities of coral-reef microorganisms, which has been motivated by the growing evidence that populations of microbes, and bacteria in particular, fundamentally shape the health and ecology of coral reef ecosystems (Krediet et al., 2013; Rosenberg et al., 2007). More specifically, a principle motivation for this thesis is to shift the focus of coral microbiological research from laboratory-based studies, to *in-situ* examinations of the behavioural mechanisms (e.g. chemotaxis) that microorganisms use to locate and colonize their coral hosts. While there is evidence from laboratory studies using cultured isolates that bacterial chemotaxis may be important in coral disease on-set (Banin et al 2001; Meron et al. 2009; Vega-Thurber et al. 2009; Kimes et al., 2011; Garren et al., 2011), this behaviour has never been documented to occur in the environment among natural populations of coral reef bacteria. To address this and other important open questions in coral reef microbiology, we have coupled a suite of techniques including *in situ* microfluidic experimentation and ecogenomic approaches to examine microbial behaviours and functional dynamics within natural coral reef ecosystems. Using this approach we have provided several new insights into coral-microbe interactions, which will be synthesized in this final chapter.

Coral-reef microbes, who are they, where are they and what are they doing?

The 1970's saw a significant focus shift towards the microbial ecology of coral reefs (DiSalvo, 1971; Sorokin, 1973) and since then the role and dynamics of the bacterial fraction of the microbial community associated with corals has become of particular focus and has included efforts to identify coral-associated bacteria and determine how they differ between coral species (Ritche and Smith, 1997; Rohwer et al., 2001; 2002; Chen et al., 2011), how they differ geographically (Littman et al., 2009; Barott et al., 2011) and how they change under different disease states or environmental perturbations (Vega-Thurber et al., 2009; Littman et al., 2011). While these studies have revealed the staggering diversity and apparent importance of coral reef bacterial communities, there have only been a few glimpses of the ecological, functional and behavioural mechanisms that under-pin coral-microbe interactions.

In chapter 1, we aimed to address these knowledge gaps in coral-microbial ecology by using metagenomics to examine the microbial ecology of a single coral reef ecosystem, whereby we measured the diversity and functional capacity of microbial communities inhabiting specific microniches within the reef, including coral-associated and non-coral associated niches throughout the reef. While two previous studies have used similar metagenomic approaches to explore patterns in microbial composition and function between chains of coral reef ecosystems (Dinsdale et al., 2008; Bruce et al., 2012), our research presented in chapter 1 reveals substantial "within" reef variability. This chapter highlighted that the presence of coral hosts significantly influences microbial community composition and functional potential within a reef, with substantially different metagenomic profiles observed in coral-associated samples to open water and sediment associated samples. Specifically, microbes associated with coral surface seawater metagenomes were indicative of a predominance of microbes with a copiotrophic life style, including members of the *Pseudoalteromonas, Alteromonas and Vibrio* genera. This observation of increased abundance of *Vibrios* is particularly notable because members of this genus have previously been identified as known coral pathogens (Banin et al 2001; Meron et al. 2009; Vega-Thurber et al. 2009; Kimes et al., 2011; Garren et al., 2011), and this is an aspect of coral reef microbiology that we re-visit in Chapters 4 and 5. In addition to microbial composition shifting dramatically to copiotrophic lifestyle, we observed an important shift in microbial metabolic potential across the different coral reef niches, whereby genes associated with more dynamic processes including chemotaxis and motility, cell signalling and regulation were in greater abundance in the coral surface sea water metagenomes, while genes typical of core "house-keeping" processes dominated the non-coral sea water metagenomes. Genes associated with bacterial motility and chemotaxis were more abundant in the coral surface sea water metagenomes and were responsible for driving significant differences between the coral and non-coral sea water metagenomes. The ability for bacteria to be motile and chemotactic on a coral reef is likely to be advantageous, enabling them to exploit chemical gradients near to the surfaces of benthic reef organsisms such as corals. The findings from Chapter 1, specifically the over-representation of chemotaxis and motility genes in the coral surface seawater metagenomes, laid the foundation for Chapter 2, where we examined if and how

natural populations of coral-bacterial communities use chemotaxis within the context of coral-microbe interactions.

How does chemotaxis regulate bacterial interactions with benthic hosts on coral reefs?

While the metagenomic results from Chapter 1 revealed that microbes associated with the surfaces of corals had an elevated relative potential for chemotaxis, with chemotaxis and motility genes responsible for driving the greatest functional differences between microbial communities within a reef, this still only provides indirect evidence for the importance of this phenotype. In Chapter 2, we addressed the potential ecological importance of chemotaxis among coral associated bacteria by performing the first *in situ* examinations of chemotaxis within a coral reef ecosystem. Indirect evidence from previous laboratory studies focussed on the processes involved in colonization and infection by coral pathogens, including *Vibrio shioli* and *Vibrio coralliilyticus,* has indicated that motility and chemotaxis may be important behaviours within the context of coral-bacteria interactions (Banin et al 2001; Meron et al. 2009; Vega-Thurber et al. 2009; Kimes et al., 2011; Garren et al., 2011). However, virtually nothing is known about how natural mixed communities of coral reef microbes may use this behaviour. The lack of *in-situ* evidence for chemotaxis in marine environments and the results obtained in Chapter 1 motivated us to conduct an investigation into coral-bacterial chemotactic behaviour. To do this we employed an *In Situ* Chemotaxis Assay (ISCA), which was designed and built using microfluidic technologies. Using both this approach and laboratory experiments conducted using the traditional capillary assay we found clear evidence for a high chemotactic capacity among coral-associated microbes. Coral associated microbes exhibited strong chemotaxis to a variety of chemicals released from the coral holobiont. Notably, chemotaxis was much stronger among populations of coral-associated, rather than non-coral associated bacteria, implying a heightened importance of this phenotype within the coral surface microenvironment. Using 16S rRNA amplicon pyrosequencing, we characterised the compositions of the chemotactic communities responding to different chemoattractants. Responding communities were typically composed of known coral-associated bacteria, but

showed substantial partitioning between different chemoattractants, indicative of a specialist response in many cases. In addition to providing the first *in-situ* evidence of chemotaxis among marine bacteria, these observations provide phenotypic confirmation of the genomic patterns observed in Chapter 1, whereby the importance of chemotaxis does indeed appear to be heightened near to the surfaces of corals. The observations made in these two chapters lead us to conclude that chemotaxis is likely to play a role in the establishment and maintenance of coral-bacteria interactions, while it can influence the community structure of these bacterial associates.

In Chapter 3, we extended upon the observations in Chapter 2, by focussing on the potential role of chemotaxis in the development of ecological associations between marine microbes and another important benthic organism inhabiting coral reefs, sponges. Chapter 3 highlights the adaptability of the ISCA to assess chemotaxis towards a new host model in the marine environment and in turn its reproducibility as an experimental platform to measure chemotaxis *in-situ*. Perhaps even more so than corals, sponges form intimate and strong associations with bacteria, whereby microbes can comprise up to 35% of the sponge's biomass (reviewed in Webster and Taylor, 2012) and 'sponge-specific' symbionts are known to only form exclusive associations with their sponge hosts (Hentschel *et al.*, 2002; Taylor *et al.*, 2007a). It has been a long held belief in sponge microbiology that the sponge host controls and maintains these microbial relationships by either (i) filtering microbes from the surrounding reef seawater (Reiswig, 1971; Webster et al., 2010) where the 'sponge-specific' symbionts are exceptionally rare (Taylor et al., 2013) and/or (ii) vertical transmission from parent sponge to offspring (Webster et al., 2010).

We demonstrate that coral reef microbes show high levels of chemotaxis towards the chemical products of sponges, implying that chemotaxis may also be important in the establishment of sponge-bacterial interactions. Notably, the phylogenetic structure of the bacterial communities exhibiting chemotaxis to sponge extracts was highly consistent with the taxa previously shown to live in association with sponges including *Piscirickettsiaceae* and *Sphingomonadaceae* (Thomas et al., 2010). Of particular interest was the occurrence of environmentally 'rare' (i.e. in the seawater) sponge-specific bacterial symbionts such as the *Gemmatimonadetes* and the

Actinobacteria, which we found in high abundance in the ISCA wells. These groups however were below detection limits in the surrounding sea water sample, indicating a substantial concentrating effect within the chemotaxis assay. The absence of these bacteria in the background seawater samples is consistent with previous research suggesting their rarity in the surrounding environment (Taylor et al., 2013).

Chapter 3 redefines the long held bacterial acquisition paradigm in sponge-microbial ecology, whereby our results have led us to propose for the first time that bacterial symbionts can actively find their sponge host on coral reefs through chemotaxis. Furthermore, these results provide additional evidence to support our hypothesis that chemotaxis is an important phenotype among coral-reef bacteria, allowing them to locate their benthic hosts.

The evidence for the importance among coral reef microbes provided by Chapters 2 and 3 is consistent with the findings of another study conducted in conjunction with this thesis work (see Appendix). In this manuscript (Garren et al. 2014), we demonstrate that the known coral pathogen *V. coralliilyticus* uses high performance chemotaxis towards the mucus of thermally stressed corals, with the coral-derived chemical dimethylsulfoniopropionate (DMSP) the putative specific chemical cue. This is consistent with our Chapter 2 findings where we show that natural populations of *Vibrio* exhibited chemotaxis towards ISCA deployments containing DMSP. Notably, the Garren et al. paper also demonstrated that DMSP concentrations increased in coral mucus under elevated seawater temperatures, and that chemotactic responses were highest to the mucus of heat stressed corals. This indicates that environmental perturbations, such as rising seawater temperatures may further enhance the importance of chemotaxis within the context of coral-microbe interactions.

Studying the well-studied: Coral-vibrios and heat stress, but not as we know it.

The results of the preceding chapters produced several lines of evidence indicating the potential ecological importance of Vibrios within the coral-microbe association. Consequently, the focus of Chapter 4 was a detailed examination of the

quantification of *Vibrio* populations among a naturally occurring mixed microbial community associated with the coral *P. damicornis* under increasing sea water temperatures.

While the influence of temperature on the infection dynamics of some pathogenic Vibrios has been examined before (Kimes et al., 2011; Garren et al., 2014), these studies have been conducted using simple single isolate systems under laboratory conditions. Chapter 4 details a quantitative report of how a temperature increase to 31°C resulted in a 2-3 orders of magnitude increase in the *Vibrio* community and a 4-order of magnitude increase in the abundance of resident *V. coralliilyticus*. The quantitative findings presented in this chapter highlight for the first time the importance of how natural populations of a known coral pathogen are able rise to prominence over other members of a mixed microbial community given the right conditions, providing further evidence that elevated sea water temperatures occurring as a result of climate change will negatively affect coral reefs by benefiting potentially pathogenic coral microbes.

Exploring the unexplored: active gene expression - what's really going on in the microbial community during thermal stress?

We further examined the potential role of environmental variability, in the form of heat stress, in shifting the influence of the chemotactic phenotype, particularly among coral pathogens in Chapters 4 and 5. Previous metagenomic research conducted by Vega-Thurber et al. (2009) provided evidence for thermal stress-driven shifts in the structure and function of coral-associated microbial communities, but we aimed to take the next step to monitor the transcriptionally active genes of the coral-microbial community in response to thermal stress.

This final chapter was a natural progression from chapter 4 given the evidence for a heat-stress induced shift in the coral microbiome towards one comprising a higher proportion of potentially pathogenic Vibrios. In chapter 5, we present a manipulative study highlighting the genes being actively expressed by the microbial community in response to increasing sea water temperatures. We emulated the 2-3°C increase in

summer maxima predicted for 2100, whereby seawater temperatures were increased to 31°C to monitor the phylogenetic and transcriptomic changes among a naturally occurring mixed microbial community associated with the coral *P. damicornis*. In Chapter 5 we used a combination of 16S rRNA sequening, metagenomics and metatranscriptomics to reveal that increases in seawater temperature led to dramatic shifts in the community composition and functional gene expression profiles. These included significant increases in the expression of chemotaxis and motility genes, which in fact were responsible for some of the largest differences between the heat stress and control treatments. These results are significant because chemotaxis and motility are recognised as key phenotypes in the coral disease process (Banin et al., 2001; Ben-Haim and Rosenberg 2002; Ben-Haim et al., 2003b; Garren et al., 2014). There is evidence that under elevated sea water temperatures the coral pathogen *V. coralliilyticus* up-regulates motility and chemotaxis genes (Kimes et al., 2011) and displays extremely fast chemotactic responses towards the mucus of thermally stressed *P. damicornis* (Garren et al., 2014), but increases in the expression of these genes have not previously been demonstrated to occur among a naturally occurring microbial population during elevated sea water temperatures. This potential significance of this observation within the context of coral disease is emphasized by our observation that a significant proportion of the up-regulated chemotaxis and motility genes were shown to be affiliated with Vibrios. Taking the results of Chapters 1, 2, 3 and 4, and our contributions to the Garren et al (2014) paper, together confirm the ecological importance of chemotaxis and motility in coral microbiomes and indicate that elevated seawater temperatures associated with climate change can lead to important behavioural shifts within the coral holobiont.

Thesis Conclusions

The research presented in this thesis has provided new insights into coral reef microbiology by demonstrating that the microbial communities within a single coral reef ecosystem are dramatically different in both community structure and function. We show that the genes associated with motility and chemotaxis can be large drivers of functional differences across microbial communities (Tout et al., 2014) and furthermore, using both laboratory and *in-situ* experiments we support these findings

by demonstrating high levels of chemotactic capacity among natural communities of coral associated bacteria (Tout et al. 2015a). Additionally, we redefined the bacterial-symbiont acquisition paradigm in sponge microbiology by showing that bacteria can actively locate their sponge host on a coral reef and taken together, our findings revealed that chemotaxis can structure the community composition of corals and sponges. Within the context of the world-wide decline of coral reefs these results are significant as we redefine how increasing sea water temperatures can dramatically alter and affect the coral-microbial community, in particular by favouring the proliferation of potential coral pathogens and enhancing the behaviours they require to successfully infect corals.

To expand upon our findings presented in this thesis, future research would benefit from focusing on exploring how bacterial chemotaxis differs among different coral species to establish whether there is any species-specific behaviour which may shed light on gaining a better insight to how coral-microbial species specific relationships are regulated. Furthermore, chemotaxis assays using cultures of known species of coral-associated bacteria and a suite of putative coral-chemoattractants could expand upon our work by unravelling the specific chemicals different species respond to. By using a suite of known coral-associated bacterial species this work would also provide insight into determining what coral-bacterial species use coral chemoattractants exclusively as chemical cues to locate their host as demonstrated with *V. coralliilyticus* (Garren et al., 2013) vs those that use them for metabolic purposes. Further work using the ISCAs with different species of sponge compounds would assist in whether or not we can begin to redefine the sponge-symbiont acquisition paradigm across the sponge-microbiology field as our research thus far only focused on one sponge species.

Overall, this thesis has contributed to expanding coral microbiology, whereby our results provide a new framework for understanding the microbial seascape of coral reefs and the mechanisms underpinning the establishment of bacterial relationships with corals and sponges, as we highlight a new role for bacteria on coral reefs whereby they can play a pivotal part in controlling interactions with benthic organisms and changes in the community composition and function can have

dramatic consequences for the health of corals during times of thermal stress as a consequence of global climate change.

Appendix

Publication arising from chapter 5

Garren M, Son K, Raina JB, Rusconi R, Menolascina F, Shapiro OH, **Tout J**, Bourne DG, Seymour JR, Stocker R (2013). A bacterial pathogen uses dimethylsulfoniopropionate as a cue to target heat-stressed corals. ISME J 1-9

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ORIGINAL ARTICLE

A bacterial pathogen uses dimethylsulfoniopropionate as a cue to target heat-stressed corals

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Diseases are an emerging threat to ocean ecosystems. Coral reefs, in particular, are experiencing a worldwide decline because of disease and bleaching, which have been exacerbated by rising seawater temperatures. Yet, the ecological mechanisms behind most coral diseases remain unidentified. Here, we demonstrate that a coral pathogen, *Vibrio coralliilyticus*, uses chemotaxis and chemokinesis to target the mucus of its coral host, *Pocillopora damicornis*. A primary driver of this response is the host metabolite dimethylsulfoniopropionate (DMSP), a key element in the global sulfur cycle and a potent foraging cue throughout the marine food web. Coral mucus is rich in DMSP, and we found that DMSP alone elicits chemotactic responses of comparable intensity to whole mucus. Furthermore, in heat-stressed coral fragments, DMSP concentrations increased fivefold and the pathogen's chemotactic response was correspondingly enhanced. Intriguingly, despite being a rich source of carbon and sulfur, DMSP is not metabolized by the pathogen, suggesting that it is used purely as an infochemical for host location. These results reveal a new role for DMSP in coral disease, demonstrate the importance of chemical signaling and swimming behavior in the recruitment of pathogens to corals and highlight the impact of increased seawater temperatures on disease pathways.

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Subject Category: Microbe-microbe and microbe-host interactions
Keywords: *Vibrio*; microfluidics; chemotaxis; DMSP; chemical signaling; motility

Introduction

The globally distributed marine bacterium *Vibrio coralliilyticus* (Pollock *et al.*, 2010) causes bleaching and tissue loss in reef-building corals (Ben-Haim *et al.*, 2003). Despite the widespread loss of corals to diseases (Harvell *et al.*, 2009), little is known about their onset, and fundamental questions, such as how a pathogen finds its host, have remained largely unanswered (Bourne *et al.*, 2009). Among human enteric pathogens, the ability to swim (motility) and guide movement in response to chemical gradients (chemotaxis) is a common phenotype in the infection process (Boin *et al.*, 2004; Croxen *et al.*, 2006). In the ocean, we found that motility is universal among putative coral pathogens (Supplementary Table S1). This prevalence, together with the presence of strong chemical gradients that can extend over 2 mm from the coral surface (Kühl *et al.*, 1995; Mass *et al.*, 2010), suggests that motile responses to chemical cues may be a pervasive mechanism for coral pathogens to locate and colonize their hosts. Yet, beyond evidence that motility and chemotaxis are involved in *Vibrio*-induced bleaching (Banin *et al.*, 2001; Meron *et al.*, 2009), there has been no direct, real-time observation of the motile behavior of pathogens, nor any insight into the specific chemical triggers of chemotaxis or its dependence on the host's physiological state. By integrating microfluidic experiments with the collection of coral exudates, we found that

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V. coralliilyticus (Pollock *et al.*, 2010) markedly changes its motility behavior in response to the mucus of its host, *Pocillopora damicornis*, to rapidly target the source of the cue.

The surface of a coral is lined with mucus of variable viscosity, which is continuously excreted for cleansing, feeding and defense (Brown and Bythell, 2005). This mucus contains a broad range of chemicals, including water-soluble glycoproteins, amino acids and metabolites (Brown and Bythell, 2005). In the mucus of many coral species, the sulfur compound dimethylsulfoniopropionate (DMSP) reaches concentrations (1–62 μM) orders of a magnitude higher than in the surrounding seawater (6–11 nM) (Broadbent and Jones, 2004; Van Alstyne *et al.*, 2006). For corals, this molecule might act as an antioxidant (Sunda *et al.*, 2002) or as an overflow system for the symbiotic zooxanthellae to excrete excess sulfur (Stefels, 2000). DMSP has also been shown to be a potent chemoattractant for several marine micro- and macro-organisms (Debose and Nevitt, 2007; Seymour *et al.*, 2010). Here, we show that DMSP is a primary chemical cue for *V. coralliilyticus*' behavioral responses to the mucus of *P. damicornis* and that its increased production under heat stress enhances the attraction of the pathogen.

Materials and methods

Organism growth conditions and laboratory mucus collection

All experiments were conducted using *V. coralliilyticus*, strain BAA-450, acquired from the American Type Culture Collection (www.atcc.org, Manassas, VA, USA) and grown in 0.2 μM filtered, autoclaved seawater (FASW) with 1% 2216 media (BD Difco) in a shaking incubator at 30°C. Small colonies of the coral *P. damicornis* (from the Birch Aquarium at Scripps, La Jolla, CA, USA) were cultured at 25 °C in artificial seawater (Instant Ocean, Spectrum Brands Company, Cincinnati, OH, USA) on a 12-h light–dark cycle. Mucus was collected from the colonies by exposing them to air for 3 min. Owing to volume requirements of the microfluidic assays, the mucus was then diluted to 1:2 in FASW and vortexed for 10 s to mix thoroughly.

Mucus collection on Davies Reef (Great Barrier Reef)

Small colonies of the coral *P. damicornis* and *Acropora millepora* were collected from Davies Reef, Great Barrier Reef, Australia (18°05′ S/147°39′ E) and transferred to the outdoor aquarium facility of the Australian Institute of Marine Science (Townsville, QLD, Australia). Mucus was collected from the colonies by removing them from the water, shaking off excess water for 10 s and then holding them upside down collecting dripping mucus with a syringe. Freshly collected mucus was then homogenized and divided in two: one half was flash-frozen in liquid nitrogen; the second half was directly extracted with 40 ml of high-performance liquid chromatography-grade methanol for DMSP quantification. The frozen portion was later used in chemotaxis assays.

Metabolism and DMSP measurements

DMSP metabolism. Two different basal media were used to determine the DMSP metabolic capabilities of *V. coralliilyticus*: a modified marine ammonium salt medium (Raina *et al.*, 2009) lacking any carbon source, and a modified basal salt medium lacking any sulfur source (Fuse *et al.*, 2000). DMSP was added to both the media (1 mM final concentration) and acted either as a sole carbon source or as a sole sulfur source. The pH was adjusted to 8.2. To account for the potential cometabolism of DMSP with other compounds present in coral mucus, mucus was collected as described above, homogenized, filtered twice (0.2 μm) and sonicated for 10 min. Five milliliters of marine ammonium salt medium, modified basal salt medium or sterile mucus were inoculated in triplicate from single *V. coralliilyticus* colonies and incubated at 28 °C between 1 and 6 days with shaking in gas-tight vials. Control bottles containing only the basal media and DMSP were used to account for the possible chemical breakdown of DMSP. Results from these experiments were confirmed using an alternative *V. coralliilyticus* strain, LMG 23696 (Sussman *et al.*, 2008). Bottles inoculated with the *Pseudovibrio* sp. P12 (an alphaproteobacterium isolated from healthy *P. damicornis*) grown under identical conditions acted as the positive control.

Acrylate metabolism. Marine ammonium salt medium lacking carbon was used to investigate the ability of strain BAA-450 to degrade acrylate (1 mM, final concentration). Five milliliters of marine ammonium salt medium were inoculated in triplicate from single BAA-450 colonies and incubated at 28 °C between 1 and 6 days with shaking. Control bottles containing only the basal medium and acrylate were set up, along with the ones inoculated with BAA-450, to account for its possible chemical breakdown. Bottles inoculated with the *Pseudovibrio* sp. P12 grown under identical conditions served as a positive control.

NMR measurements. DMSP metabolism assays and DMSP quantification were performed by ^{1}H nuclear magnetic resonance (NMR) (Tapiolas *et al.*, 2013). Briefly, the headspace of each gas-tight vial was first sampled with a syringe. Methanol (40 ml) was then added to each culture tube to extract DMSP and acrylate, and the mixtures were subsequently dried *in vacuo* using a rotary evaporator (Buchi, Flawil, Switzerland). The dried extracts were resuspended in a mixture of deuterated methanol (CD_3OD, D 99.8%, 750 μl) and deuterium oxide (D_2O, D 99.8%, 250 μl) (Cambridge Isotope

Laboratories, Andover, MA, USA). A 750-µl aliquot of the particulate-free extract was transferred into a 5-mm Norell tube (Norell Inc., Landisville, NJ, USA) and analyzed immediately by ^{1}H NMR. Spectra were recorded on a Bruker Avance 600 MHz NMR spectrometer (Billerica, MA, USA) with a TXI 5 mm probe and quantification was performed using the Electronic REference To access *In vivo* Concentrations method (ERETIC) (Tapiolas *et al.*, 2013). No DMSP degradation, acrylate by-products or dimethylsulfide smell were present in the DMSP medium experiments for *V. coralliilyticus* or the negative control, whereas all were present in the *Pseudovibrio* positive control. In the acrylate medium experiments, acrylate was degraded by both *V. coralliilyticus* and the *Pseudovibrio* positive control, but not the no-bacteria negative control. The same NMR protocol was also used to quantify the amount of DMSP present in coral mucus from *P. damicornis* and *A. millepora*.

Chemotactic index (I_C)

We quantified the magnitude of the chemotactic response using the chemotactic index, I_C, which measures the enhancement in the cell concentration within the region initially occupied by the mucus (that is, the central band in Supplementary Figure S1), relative to the cell concentration outside that area, minus 1. $I_C = 0$ thus corresponds to a uniform cell distribution (that is, no chemotaxis). See Seymour *et al.* (2010) for more details. For each experiment, triplicate 0.2 µm FASW control trials were run first, wherein the same FASW used to grow the cells and to make the DMSP dilutions was injected into the microfluidic device in lieu of an attractant. All I_C curves for a given attractant were normalized to their FASW control by subtracting the mean I_C among the three FASW trials.

To compare the I_C values observed in this study with values observed by Stocker *et al.* (2008) for *Escherichia coli* and *Pseudoalteromonas haloplanktis* in a similar (but not identical) experimental setup, data were extrapolated from Figure 2b of that manuscript and converted from the hot spot index (H) to I_C. The hotspot index was defined by Stocker *et al.* as the mean concentration of bacteria within the central, $W_C = 300$ µm wide region of the microchannel relative to the mean concentration over the entire channel width, $W = 1200$ µm. The data were converted to I_C using the following conversion formula: $I_C = ((W - W_C)/((W/H) - W_C)) - 1$.

Diffusive gradient microfluidic experiments

Microinjector device for chemotaxis assays. A 2.8-mm wide microchannel with a 400-µm wide injector (Supplementary Figure S1) was fabricated using soft lithography techniques described previously (Seymour *et al.*, 2008) to establish diffusive gradients for chemotaxis assays. Briefly, the attractant was injected into the microchannel (Supplementary Figure S1; inlet B) as a 400-µm wide band equidistant from the channel's side walls, whereas the cells were injected in the channel on either side of the band (Supplementary Figure S1; inlet A). The cells and attractant were flowed into the channel and then the flow was stopped to allow the attractant to diffuse laterally and the cells to respond to the gradient. DMSP (DMSP · HCl; C5H10SO2 · HCl; TCI) was freshly prepared with FASW to make 15 µM, 45 µM and 61 µM working solutions that closely corresponded to the amount of DMSP measured in the *P. damicornis* and *A. millepora* mucus samples. *A. millepora* was chosen as a second species to test because *V. coralliilyticus* is known to infect it as well (Sussman *et al.*, 2009). These freshly prepared DMSP solutions as well as *P. damicornis* mucus collected from Davies Reef (Great Barrier Reef; preserved at −80 °C (as described in the mucus collection section) and thawed on ice directly before experimental use; measured to contain 12–15 µM DMSP) and from corals maintained in the laboratory at MIT, *A. millepora* mucus from the Great Barrier Reef (containing 45–62 µM DMSP) and a FASW control were tested against overnight cultures of *V. coralliilyticus*.

The channel was loaded at moderate flow rates (2 µl min^{-1}) to establish an initial experimental condition where the cells and the attractant were in discrete bands (Supplementary Figure S1B). To begin the experiment, the flow was stopped and the channel was imaged directly downstream of the end of the microinjector using phase-contrast video microscopy on a Nikon Ti microscope (Tokyo, Japan) equipped with an Andor Neo CCD camera (6.5 µm/pixel; Belfast, UK) at 1 frame per second for 6 min. Five replicates of each experiment were conducted, and the microchannel was flushed for 30 s with fresh cells and attractant between replicates. Flushing with FASW lasted for 2 min in between different attractants. Each video was analyzed for cell positions using an automated image segmentation software developed in-house with MATLAB (MathWorks, Natick, MA, USA). Background subtraction and cross-correlation functions were used to detect non-motile cells or other particles from the mucus, which were excluded from the cumulative cell distribution across the channel. The resulting time series of cell distributions are presented for *P. damicornis* (Figure 1) and for *A. millepora* (Supplementary Figure S2).

Temperature stress experiment on Heron Island

To test the response of *V. coralliilyticus* to mucus from corals under high-temperature stress, a field experiment was carried out on Heron Island, Great Barrier Reef, Australia (23°26′ 37″S/151°54′ 44″E). Three colonies of *P. damicornis* were collected from the reef flat in front of the Heron Island Research

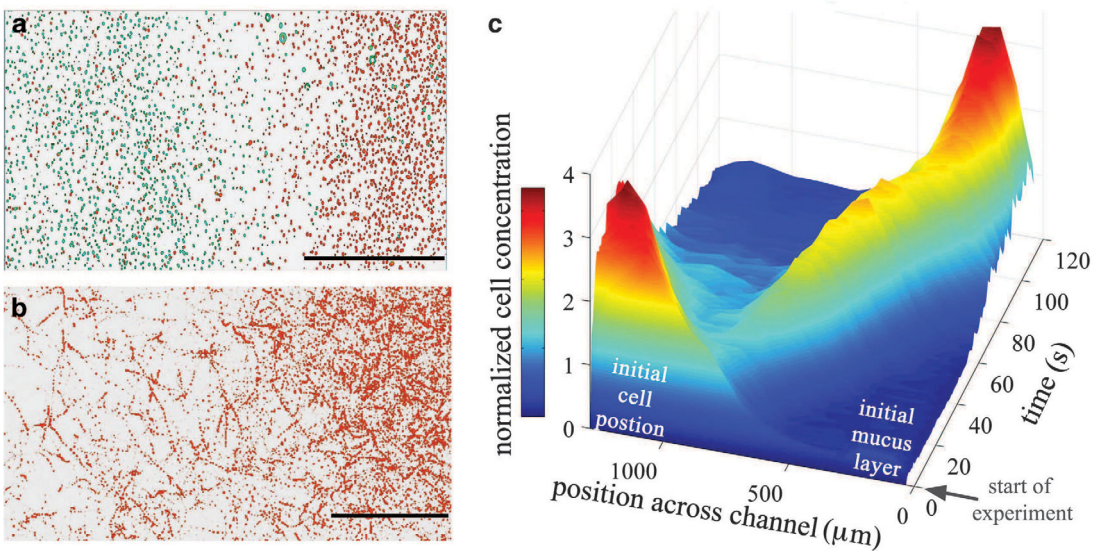

Figure 1 *V. coralliilyticus* is strongly attracted to coral mucus. (**a**) Positions and (**b**) trajectories of individual *V. coralliilyticus* cells exposed to a diffusing coral mucus gradient in a microfluidic channel (Supplementary Figure S1). A 400-μm thick layer of mucus, harvested from laboratory-cultured *P. damicornis* corals, was created in a microchannel (half of the layer is shown) and allowed to diffuse. The scale bars are 200 μm. In (**a**), cell positions at the start of the experiment and after two minutes are colored teal and red, respectively, and overlaid. In (**b**), trajectories acquired between 100 and 115 s after the start of the experiment are shown. The two panels show the strong shift in the cells' position and their intense accumulation into the mucus layer (the right side of the images). Also see Supplementary Movie S1. (**c**) The full time series of the spatial distribution of the pathogen population across the width of the microfluidic channel. Color and height both measure the local, instantaneous concentration of bacteria, normalized to a mean of one. Note the intense wave of bacteria actively migrating into the mucus layer.

Station, fragmented into 48 nubbins and allowed to recover and acclimate in a flow-through seawater tank pulling water from the reef flat for 8 days. Fragments were then distributed evenly into six tanks with three fragments from each donor colony in each tank. A randomized sample design for both fragment placement within the tanks and treatment assignment to each tank was employed. Three tanks were maintained at ambient seawater temperature (22 °C) for the duration of the experiment, and the other three tanks began at ambient temperature and then were slowly ramped by 1.5 °C per day for 7 days. All fragments were sampled for mucus by air exposure as described above at the initial time and after 7 days, when the temperature-treated tanks reached 31 °C. One-third of the mucus samples were preserved for DMSP measurements (described above) by adding 600 μl of methanol and freezing at −20 °C. Clonal replication is essential for comparing responses because DMSP concentration can vary with irradiance, zooxanthellae density and seawater temperature (Sunda *et al.*, 2002; Van Alstyne *et al.*, 2006). The rest of the samples were immediately frozen at −80 °C unaltered and shipped to MIT, where they were used in microfluidic chemotaxis experiments with the microinjector setup (Supplementary Figure S1). Replicate mucus samples from the heat-stress experiment were tested on three different days in the lab with freshly grown *V. coralliilyticus* cells. All trials yielded comparable results to those shown in the main text (Figure 2b; Supplementary Figure S6).

Mathematical model of simultaneous chemotaxis and chemokinesis

We modeled the chemotaxis and chemokinesis of *V. coralliilyticus* using an existing modeling framework for bacterial chemotaxis (Brown and Berg, 1974; Jackson, 1987; Kiørboe and Jackson, 2001), augmented by a concentration-dependent swimming speed that was based on our experimental observations (Figure 3a; Supplementary Information; Supplementary Figures S4 and S9). For simplicity and based on results from Figure 3a, we modeled chemokinesis as a 24% increase in swimming speed, from 66 μm s^{-1} at a relative chemoattractant concentrations of $C \leqslant 20\%$ of pure attractant to 82 μm s^{-1} for $C > 20\%$. From the spatial distribution of 3000 cells across the channel at each time point, we computed the time series of I_C (as detailed in the chemotactic index section of Materials and Methods). This was done twice: once in the presence of chemokinesis, and once in the absence of chemokinesis, in which case the swimming speed was uniformly equal to 66 μm s^{-1}. Results are presented in Figure 3b.

Results and discussion

To examine the ecological mechanism behind coral infection by *V. coralliilyticus*, we performed chemotaxis experiments using a microfluidic assay. *V. coralliilyticus* responded to coral mucus with remarkable speed and directionality. A microfluidic

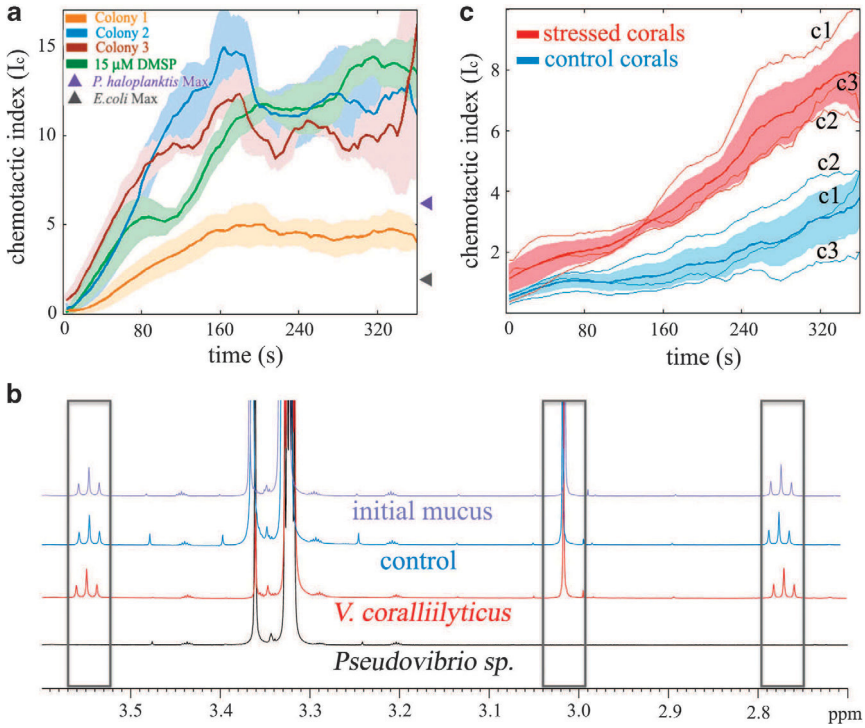

Figure 2 The pathogen's chemotaxis is primarily triggered by DMSP and is enhanced by heat stress of the host. (**a**) Time series of the chemotactic index, I_C, (a measure of the strength of cell accumulation) of *V. coralliilyticus* in response to a 400-µm thick layer (Supplementary Figure S1) of coral mucus (Colonies 1–3) or 15 µM DMSP (green line). Solid lines and shading represent the mean and s.e. of three replicate experiments. Mucus was collected from three different colonies of *P. damicornis* on Heron Island and contained 11.9–14.8 µM DMSP (Supplementary Information). The pathogen responds with comparable intensity to DMSP and mucus. Shown for reference are also the maximum chemotactic indices (suitably converted from Stocker *et al.* (2008) attained over 15 min by *E. coli* responding to a mixture of two of its most potent chemoattractants at near-optimal concentrations (serine and aspartate, 10 µM each; gray triangle) and by *P. haloplanktis* responding to algal exudates (purple triangle). All data were normalized against the respective no-attractant controls. (**b**) Profiles from quantitative NMR of freshly collected *P. damicornis* mucus from Heron Island (initial mucus, purple) reveal distinct peaks for DMSP (gray boxes). Twenty-four hours of incubation of whole mucus with *V. coralliilyticus* (red) resulted in no measurable DMSP degradation, akin to the no-bacteria control (blue), whereas the positive control strain *Pseudovibrio spp.* degraded DMSP entirely, as evidenced by the disappearance of the DMSP peaks (black). (**c**) Time series of *V. coralliilyticus*' I_C, in response to coral mucus from a clonally replicated temperature-stress experiment (maximum = 31 °C) performed on Heron Island. Chemotaxis was twice as strong toward mucus from stressed coral fragments (red) as compared with fragments from the same colonies maintained at ambient temperature (blue). Thin lines show each of the three individual colonies (c1–c3), bold lines show their mean and shading represents the s.e. All curves were normalized against seawater controls.

device was used to create a 400-µm thick layer of mucus adjacent to a 1-mm thick seawater suspension of *V. coralliilyticus*, and we imaged the spatial distribution of the pathogen population with high-temporal-resolution (15 frames s^{-1}) video microscopy (Figure 1; Supplementary Figure S1). Within 10 s of exposure to the mucus, bacteria began swimming up the associated chemical gradient. Within 60 s, >50% of cells had migrated into the 400-µm thick layer of mucus (Figure 1; Supplementary Movie S1). We quantified the magnitude of the pathogen's chemotactic response with the chemotactic index, I_C (Seymour *et al.*, 2010), which measures the enhancement in cell concentration within the initial mucus layer relative to the cell concentration outside that layer ($I_C = 0$ corresponds to no chemotaxis). *V. coralliilyticus* reached $I_C > 14$ within 3 min of being exposed to mucus (Figure 2a), a much more intense response than previously observed for either enteric or marine bacteria (Figure 2a). The strength of this response was confirmed by tracking individual bacteria and quantifying their mean chemotactic velocity (Supplementary Figures S3–S5), which reached 36% of the average swimming speed, considerably higher than the 5–15% typical of the model organism for bacterial chemotaxis, *E. coli* (Ahmed and Stocker, 2008).

To determine the chemical signal responsible for this response, we analyzed coral mucus using quantitative NMR (Tapiolas *et al.*, 2013). The DMSP concentrations in mucus from healthy colonies collected on Davies Reef, were high, ranging from 11.9–14.8 (±1.2) µM for *P. damicornis* and up to 62.2 (±2.0) µM for *A. millepora*, another coral species susceptible to *V. coralliilyticus* infection (Sussman *et al.*, 2009). Additional chemotaxis experiments revealed that DMSP (15 µM), when used as the sole attractant, elicited a chemotactic response of comparable magnitude to *P. damicornis* mucus ($I_{C,MAX} \sim 14$; Figure 2a). The pathogen's response varied somewhat from colony to colony, as is

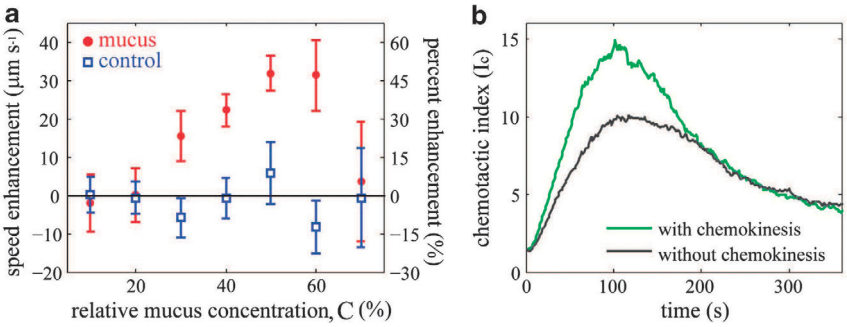

Figure 3 *V. coralliilyticus* exhibits chemokinesis that markedly increases the strength and speed of its response to coral mucus. (**a**) Swimming speed enhancement as a function of the mucus concentration instantaneously experienced by cells (Supplementary Figure S3). The enhancement is relative to the mean swimming speed in the absence of mucus (66 μm s^{-1}) and is expressed as both a speed difference (left axis) and a percent difference (right axis). Mucus concentrations, predicted from solution of the diffusion equation (Supplementary Information), are expressed relative to full mucus (i.e., the initial mucus concentration in the microchannel). Error bars represent the s.e. (**b**) A mathematical model of bacterial motility (Supplementary Information) shows that $I_{C,MAX}$ is 50% higher when chemokinesis in included (green) compared with the case of chemotaxis alone (gray), and that $I_{C,MAX}$ without chemokinesis is reached in <50% of the time when chemokinesis is present. Chemokinesis was modeled as a 24% enhancement of the mean swimming speed in regions with mucus concentrations greater than 20% of full mucus, based on the observed speed–concentration relationship (panel **a**).

expected owing to the natural variability in mucus composition among colonies. This variability notwithstanding, all responses observed with DMSP at comparable concentrations to those found in coral mucus were consistent with it being a primary driver of the chemotaxis. In addition, all responses observed were substantially faster and stronger than previously observed chemotactic responses of the enteric bacterium *E. coli* to its strongest chemoattractants and of the marine bacterium *P. haloplanktis* to algal exudates (Figure 2a). The same was true for *A. millepora* mucus when chemotactic responses to DMSP alone in mucus-equivalent concentrations were compared with responses to whole mucus (Supplementary Figure S2). These results demonstrate that DMSP within coral mucus is a major driver of the pathogen's behavior.

Chemotaxis by marine bacteria toward DMSP has previously been ascribed to DMSP's value as a rich carbon and sulfur source, as bacteria may obtain up to 15% of their carbon and most of their sulfur from DMSP (Zubkov *et al.*, 2001). Intriguingly, despite its vigorous swimming response toward DMSP, *V. coralliilyticus* does not detectably metabolize the compound. NMR analysis following a 6-day incubation of the pathogen in minimal media, where DMSP was either the sole carbon or the sole sulfur source, showed no degradation of DMSP by *V. coralliilyticus* (Supplementary Figure S7). The bacterium similarly did not degrade DMSP within whole-coral mucus (Figure 2b), confirming that it cannot cometabolize DMSP with other mucus-derived molecules. *V. coralliilyticus*' inability to metabolize DMSP, combined with its lack of genes homologous to any known DMSP degradation gene (Supplementary Table S2), indicates that DMSP is used by this pathogen purely as an infochemical, a function that DMSP and its derivatives also serve among pelagic reef fish (Debose and Nevitt, 2007) and marine protists (Seymour *et al.*, 2010; Garcés *et al.*, 2013). By demonstrating that the origin of the DMSP cue for *V. coralliilyticus* is its host, these results provide the first evidence that DMSP has a signaling role in the onset of a bacterial disease by aiding in the detection of a suitable host.

The importance of DMSP as a signaling cue, together with evidence that stressed corals are more susceptible to bacterial disease (Bruno *et al.*, 2007), prompted us to examine whether the mucus of stressed corals contains more DMSP and elicits stronger responses in *V. coralliilyticus* compared with unstressed controls. We performed a clonally replicated high-temperature stress experiment (temperature raised 1.5 °C/day over 6 days) on Heron Island. Analysis by quantitative NMR demonstrated a fivefold increase in DMSP concentration in the mucus of heat-stressed *P. damicornis* fragments (31 °C) compared with control fragments from the same colonies (maintained at 22 °C). No other molecule was substantially enriched in the stressed coral mucus compared with the controls, with the exception of DMSP's main degradation product, dimethylsulfide, toward which *V. coralliilyticus* chemotaxes only very weakly (Supplementary Figure S8). This result shows that DMSP may therefore be a strong cue for *V. coralliilyticus* to detect stressed or susceptible coral hosts. This latter hypothesis was supported by further chemotaxis experiments, which yielded a twofold higher I_C in response to mucus from stressed fragments ($I_{C,MAX} = 8$) relative to mucus from control fragments ($I_{C,MAX} = 4$) (Figure 2c; Supplementary Figure S6). The stress-induced enhancement of host-released cues and the simultaneous intensification of the pathogen's response strongly suggest that chemical signaling and active behavior represent important components in the pathway to disease. Furthermore, the widespread increase in susceptibility to

bacterial infection of stressed animals (Mydlarz *et al*., 2006; Verbrugghe *et al*., 2012) suggests that chemical interactions such as these may be a recurring element in other marine diseases.

The behavioral response of *V. coralliilyticus* to coral mucus and DMSP is not limited to chemotaxis, but includes a second, powerful behavioral adaptation: chemokinesis. Whereas chemotaxis is the ability to bias swimming direction in response to a chemical gradient, chemokinesis is the ability to change swimming speed in response to a change in chemical concentration. By analyzing trajectories of individual swimming cells (Supplementary Figure S3), we found that *V. coralliilyticus* exhibits a strong chemokinetic response to coral mucus (Supplementary Figures S4 and S9). *V. coralliilyticus* increases its mean swimming speed by 24% (from $66\,\mu m\,s^{-1}$ to $82\,\mu m\,s^{-1}$) in regions with $C > 20\%$ (where C is the local mucus concentration as a percentage of full mucus) and by up to 48% ($98\,\mu m\,s^{-1}$) where $C \approx 60\%$ (Figure 3a). At even higher concentrations, cells slowed down ($70\,\mu m\,s^{-1}$) possibly to retain their position near the source.

The rare ability of *V. coralliilyticus* to simultaneously employ chemotaxis and chemokinesis represents a powerful adaptation for responding to the chemical signals emanating from its coral host. Chemokinesis alone would result in the dispersion of cells away from regions of high chemical concentration because faster swimming cells have higher dispersal rates (Schnitzer, 1990). However, a mathematical model (Supplementary Information) reveals that, when paired with the ability to sense gradients, chemokinesis can substantially increase the chemotactic velocity and therefore reduce the time required to traverse a chemical gradient. A modest concentration-dependent increase in swimming speed (24%) nearly halved the timescale of the response to mucus (55 s vs 105 s to reach $I_C = 10$) and increased the peak response intensity by 50% ($I_{C,MAX}$ of 15 vs 10; Figure 3b; Supplementary Information). Although chemokinesis has been observed in a range of bacteria (Barbara and Mitchell, 2003; Seymour *et al*., 2010), neither its disproportionate contribution to a bacterium's ability to climb chemical gradients nor its potential importance in an infection process have been previously reported. This observation indicates that chemokinesis is a powerful behavioral adaptation in *V. coralliilyticus*'s response to host-derived chemical signals and that bacterial infection of corals may be driven by considerably more specific adaptations than a binary presence or absence of motility (Meron *et al*., 2009).

If DMSP represents a steady signal that seemingly provides ample time to locate the coral surface, why does *V. coralliilyticus* exhibit multiple, energy-intensive (Taylor and Stocker, 2012) motility adaptations of such magnitude? We hypothesize that the reason lies in the highly dynamic environment at the coral surface, where resident microbes contend for the best niches (Ritchie, 2006), mucus is periodically shed (Garren and Azam, 2012), surface cilia deter the attachment of fouling organisms (Wahl *et al*., 1998) and external flows on the order of millimeters per second sweep over the colony (Lesser *et al*., 1994). The residence time next to the surface is likely to be short, offering the pathogen only limited windows of opportunity for colonization of the host. In this complex environment, the ability to swim with high directionality (chemotaxis) and accelerate when accelerate when heightened concentrations are detected (chemokinesis) could aid *V. coralliilyticus* substantially in reaching the coral mucus.

Once a cell is within the mucus layer, it is only a short distance away from the coral tissue, and mucus itself is unlikely to slow its progress toward the tissue. This is supported by mathematical models (Spagnolie *et al*., 2013) that predict that even when the medium is viscoelastic and has twice the viscosity of seawater, a bacterium with a single polar flagellum, such as *V. coralliilyticus*, will actually experience a minor increase (<5%) in swimming speed. The mucus layer is furthermore often very thin (a few hundred micrometers; Jatkar *et al*., 2009), including for *P. damicornis* (Garren and Azam, 2012), requiring only seconds to traverse at typical swimming speeds. Finally, our experiments on chemokinesis, conducted with a 333 μm thick layer of whole mucus, showed no evidence

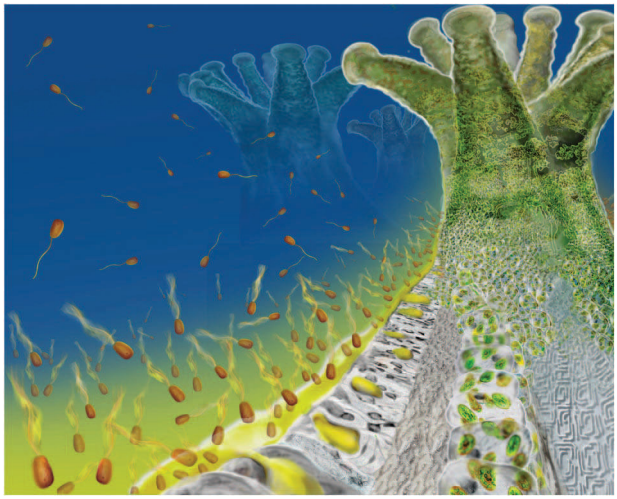

Figure 4 A new model for host detection by coral pathogens. The coral surface represents an intense source of molecules, such as DMSP, that diffuse (yellow gradient) away from the surface and out into the surrounding water, thereby establishing chemical gradients that motile bacterial pathogens (not to scale) can use to navigate toward their host. The striking prevalence of motility among putative coral pathogens (Supplementary Table S1), together with the strength of the chemical signals at the coral surface relative to typical signals in the water column, indicate that the advanced motility adaptations described here could be a widespread phenotype associated with coral disease.

for any decrease in motility. Rather these experiments demonstrated an up to 48% increase in speed at intermediate mucus concentrations. As a result, we expect that bacterial pathogens will have no physical difficulty penetrating the mucus layer.

Our findings reveal a previously unrecognized role of DMSP in the pathway to coral disease. The discovery that DMSP is involved in a pathogen's response to its host broadens the diversity of roles that this molecule has in the ocean (Kirst, 1989; Kirst *et al.*, 1991; Stefels, 2000; Sunda *et al.*, 2002; Debose and Nevitt, 2007; Simó *et al.*, 2009; Seymour *et al.*, 2010) to include that of a kairomone (a chemical that benefits the receiving but not the producing organism) and, together with the recent observation of a marine parasitoid using dimethylsulfide to locate its dinoflagellate host (Garcés *et al.*, 2013), suggests that sulfur compounds may be involved in host recognition in a broader class of marine infections. The surprising observation that *V. coralliilyticus* was unable to degrade DMSP, which is otherwise a rich carbon and sulfur source for bacteria (Zubkov *et al.*, 2001), suggests that the pathogen either has an unknown pathway for utilizing DMSP at exceedingly slow rates or it uses DMSP solely as a strong infochemical—our metabolic analyses point toward the latter explanation. The finding that hosts under heat stress exude mucus that is richer in DMSP and triggers heightened pathogen responses indicates that *V. coralliilyticus* may use this chemical cue to target stressed hosts. This emphasizes the risks posed to corals by warming waters and contributes to understanding the mechanisms underlying disease outbreaks associated with increasing seawater temperatures. Taken together, these observations unveil a strong role of microscale chemical ecology and microbial behavior in coral disease (Figure 4), emphasizing that the mechanistic drivers of many ecosystem processes may best be understood at the microscale level.

Conflict of Interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Temperature-induced behavioral switches in a bacterial coral pathogen

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Evidence to date indicates that elevated seawater temperatures increase the occurrence of coral disease, which is frequently microbial in origin. Microbial behaviors such as motility and chemotaxis are often implicated in coral colonization and infection, yet little is known about the effect of warming temperatures on these behaviors. Here we present data demonstrating that increasing water temperatures induce two behavioral switches in the coral pathogen *Vibrio coralliilyticus* that considerably augment the bacterium's performance in tracking the chemical signals of its coral host, *Pocillopora damicornis.* Coupling field-based heat-stress manipulations with laboratory-based observations in microfluidic devices, we recorded the swimming behavior of thousands of individual pathogen cells at different temperatures, associated with current and future climate scenarios. When temperature reached $\geqslant$23 °C, we found that the pathogen's chemotactic ability toward coral mucus increased by $>$60%, denoting an enhanced capability to track host-derived chemical cues. Raising the temperature further, to 30 °C, increased the pathogen's chemokinetic ability by $>$57%, denoting an enhanced capability of cells to accelerate in favorable, mucus-rich chemical conditions. This work demonstrates that increasing temperature can have strong, multifarious effects that enhance the motile behaviors and host-seeking efficiency of a marine bacterial pathogen.
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Introduction

Although disease is a natural process in any ecosystem, the influence of a changing climate on host–pathogen interactions has the potential to markedly shift the incidence of disease and thus alter ecosystem structure and functions in unpredictable ways (Burge *et al.*, 2014). Within coral reef ecosystems there is growing evidence that coral diseases are most prominent during the warmest months of the year and that corals are losing their seasonal reprieve from disease advancement as mean winter temperatures rise (Weil *et al.*, 2009; Heron *et al.*, 2010; Case *et al.*, 2011; Burge *et al.*, 2014). However, it remains unclear whether this pattern occurs as a consequence of increased coral susceptibility above specific thermal thresholds, shifts in the behavior or virulence of the responsible pathogens, or a combination of both factors.

Coral reefs around the globe are declining due to a confluence of factors, including increased disease and rising ocean temperatures (Hoegh-Guldberg 2004; Pandolfi *et al.*, 2003; Ruiz-Morenol *et al.*, 2012). Although we know that elevated seawater temperatures destabilize the coral–algal symbiosis that is critical for coral survival (Baker *et al.*, 2008), we know far less about the linkages between warming waters and coral disease. Understanding how pathogen navigation and host-detection are influenced by changing environmental conditions is important to allow these processes to be accounted for in understanding coral heath and in forecasting areas of high risk for disease outbreaks (Maynard *et al.*, 2015).

Several bacterial diseases of corals have been linked to increases in seawater temperatures, yet the mechanisms by which warmer temperatures favor disease processes remain elusive. For example, the bacterium *Vibrio shiloi* causes only slow partial bleaching at 23 °C, but rapid and severe bleaching at 29 °C in the Mediterranean coral *Oculina patagonica* (Toren *et al.*, 1998; Banin *et al.*, 2001). Similarly, the globally distributed bacterial pathogen *Vibrio coralliilyticus* (Pollock *et al.*, 2010) causes rapid

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tissue lysis of its coral host, *Pocillopora damicornis*, when temperatures exceed 26 °C (Ben-Haim and Rosenberg 2002; Ben-Haim *et al*., 2003) and is more abundant in heat-stressed corals (Tout *et al*., 2015b), whereas increases in Caribbean Yellow Band Disease between 1998 and 2010 have been associated with elevated winter seawater temperatures (Burge *et al*., 2014; Weil *et al*., 2009).

Recently, we demonstrated that *V. coralliilyticus* uses two specific behaviors to navigate toward its host: the ability to swim up chemical gradients (chemotaxis) and the ability to increase swimming speed in the presence of coral mucus (chemokinesis). Furthermore, we found that the concentration of an important chemical signal in that process, the sulfur compound dimethylsulfoniopropionate, is higher when the coral hosts are heat-stressed (Garren *et al*., 2014). This evidence suggests that elevated temperatures may improve the pathogen's capacity to locate and colonize its host owing to increased signal production. However, the effects of temperature directly on the pathogen's host-sensing behaviors are unknown. Although warmer temperatures might increase the chance of infection in several ways, such as increasing bacterial growth rates or virulence (Kimes *et al*., 2012), the effect of temperature on pathogen motility appears particularly important because all putative bacterial pathogens of corals that have been identified thus far are motile (Garren *et al*., 2014), with both motility and increased seawater temperatures independently implicated in the infection process (Banin *et al*., 2001; Meron *et al*., 2009).

Temperature has been shown to influence motility and chemotaxis in enteric and marine bacteria. *Escherichia coli* increases its average swimming speed almost linearly with temperature, from 15 $\mu m\,s^{-1}$ at 20 °C to 55 $\mu m\,s^{-1}$ at 40 °C, yet faster swimming does not correspond to stronger chemotaxis (Maeda *et al*., 1976). Among marine bacteria, two temperate *Vibrio* species increase their swimming speed with temperature: *Vibrio anguillarum* increases its speed from 25 $\mu m\,s^{-1}$ at 5 °C to 40 $\mu m\,s^{-1}$ at 25 °C (Larsen *et al*., 2004) and exhibits stronger chemotaxis at the warmer temperatures within this range, whereas *Vibrio alginolyticus* increases its speed from 77 $\mu m\,s^{-1}$ at 25 °C to 116 $\mu m\,s^{-1}$ at 35 °C (Magariyama *et al*., 1995). Albeit based on relatively low-resolution quantification of motility (tracking of small cell numbers) and chemotaxis (via the capillary assay), these observations highlight the important role that temperature can have on bacterial motility and chemotaxis. It is currently unknown how the changing environmental conditions in coral reef ecosystems will influence the behavior and ecology of bacterial pathogens that affect regional and global-scale coral health. Here, we quantify the effect of temperature on *V. coralliilyticus'* navigational performance toward mucus from its coral host as an important step in predicting the effects of warming seawater temperature on coral disease outbreaks.

Materials and methods

Two sets of experiments were conducted to (i) determine the effect of temperature on pathogen behavior and to (ii) test the simultaneous and combined effect of temperature on host and pathogen. The first set of experiments was conducted by growing the coral pathogen, *V. coralliilyticus,* at four temperatures (20 °C, 23 °C, 27 °C and 30 °C) spanning the range of seasonal mean temperatures experienced by this host–pathogen pair and include two temperatures (23 °C and 27 °C) that straddle the previously determined 26 °C trigger point for increased virulence (Ben-Haim *et al*., 2003; Kimes *et al*., 2012). We explored the bacterium's chemotactic and chemokinetic responses at these four temperatures by using a single, homogenized pool of coral mucus collected from laboratory-cultured corals growing at a moderate temperature (25 °C). The second set of experiments coupled pathogen growth at ambient and high temperatures (22 °C and 30 °C) with mucus from field-collected corals before (22 °C) and after a heat-stress (31 °C) treatment to test the simultaneous effect of temperature on the host–pathogen interactions.

Organism growth conditions

Small colonies of the coral *P. damicornis* were cultured in the laboratory at 25 °C ± 1 °C in artificial seawater (Instant Ocean, Spectrum Brands Company, Cincinnati, OH, USA) on a 12 h light–dark cycle. All experiments were conducted using the bacterium *V. coralliilyticus*, strain BAA-450, acquired from the American Type Culture Collection (www.atcc.org, Manassas, VA, USA). In the first set of experiments, cells were grown for 16–18 h, shaking (300 rpm), in 0.2 µm-filtered, autoclaved seawater with 1% marine broth (2216; BD Difco, Franklin Lakes, NJ, USA) at each of four temperatures: 20 °C, 23 °C, 27 °C and 30 °C. For growth below room temperature (20 °C and 23 °C), a chilling incubator (Multi-Therm, Denville Scientific, South Plainfield, NJ, USA) was used. Given the availability of only one chilling incubator, experiments were run back to back on subsequent days with 30 °C treatments repeated on each day as a standard: all replicates for 20 °C, 27 °C and 30 °C were performed on day 1 and all replicates for 23 °C and 30 °C were performed on day 2. Quantifications of the strength of chemotactic accumulation, measured by a normalized chemotactic index I_C (see below) for each temperature, were normalized to the 30 °C experiment from each given day and are consistently reported as the mean of all replicates (three technical replicates for each of three biological replicates). Three replicate cultures were grown at each temperature each day, all from the same three starting colonies streaked from glycerol stocks onto marine broth agar plates. The same starting colonies for a given experiment were used on both days to ensure

that every temperature had identical biological replicates. In the second set of experiments using mucus collected in the field (see below), bacteria were grown at temperatures corresponding to the ambient (22 °C) and heat-stressed (30 °C) coral conditions, again using the same three starting colonies to initiate the biological replicates for both temperatures.

Mucus collection

For the first set of experiments, designed to isolate the effect of temperature on pathogen behavior, mucus was collected in the laboratory from coral colonies cultured at 25 °C by exposing them to air for 3 min over a sterile 50 ml tube (Falcon, Corning Life Sciences, Tewksbury, MA, USA). Owing to the volume requirements for completing all microfluidic experiments with a homogeneous pool of mucus, collection was carried out on each of four colonies once per day for 5 days, and mucus was stored at – 80 °C. Directly before the experiment, a single homogenized pool of mucus was created by thawing all of the frozen mucus (all colonies, all days), pooling samples in a single 50 ml tube, vortexing for 2 min and subsequently using the pooled sample in microfluidic experiments.

In the second set of experiments, designed to test the simultaneous and combined effect of temperature on host and pathogen, we assayed chemotaxis (see below) of pathogen cells grown at one of two temperatures (22 °C and 30 °C) to mucus from coral fragments before ($T_0 = 22$ °C) and after ($T_f = 31$ °C) high-temperature stress conditions. A field experiment carried out on Heron Island, Great Barrier Reef, Australia (23° 26' 37" S/151° 54' 44" E) to obtain these mucus samples was described previously (Garren *et al*., 2014). In brief, clonally replicated, 1-week duration heat-stress experiments were performed on Heron Island using *P. damicornis*. Mucus was collected from each fragment at the beginning (temperature T_0) and end (temperature T_f) of the experiment by air exposure, as described above. Samples were immediately frozen at – 80 °C, shipped to MIT on dry ice and subsequently used in microfluidic experiments.

Microfluidic chemotaxis experiments

To test the chemotactic response of *V. coralliilyticus*, we used a 600 µm-wide, 100 µm-deep microfluidic channel with three inlets (Figure 1a) to establish a transient chemical gradient driven solely by molecular diffusion. The device was fabricated using soft lithography techniques described previously (Weibel *et al*., 2007; Seymour *et al*., 2008), bonded to a microscope slide, and mounted onto a Nikon Ti microscope (Tokyo, Japan). All experiments were conducted at the same temperature at which the cells were grown, using a temperature controlled stage insert (Warner Instruments, Hamden, CT, USA). Cell

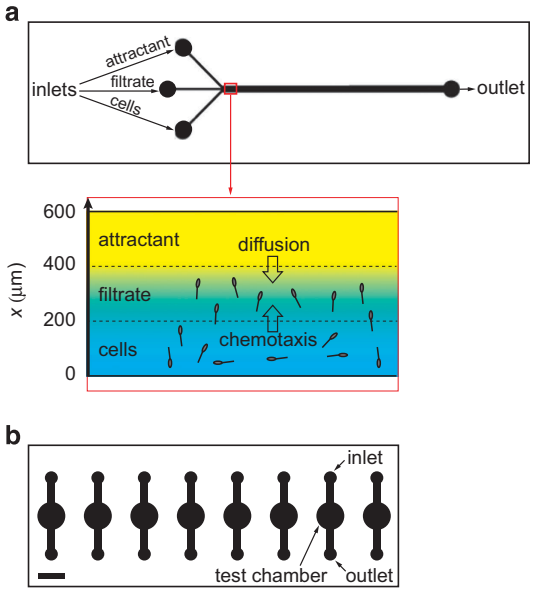

Figure 1 Schematics of the microfluidic devices used to study the temperature dependence of motility behaviors of the coral pathogen *Vibrio coralliilyticus*. Both panels presented from the microscopist's perspective, where the objective is directly below the image shown and the devices are mounted onto a standard 1 × 3 inch microscope slide. (**a**) Planar view of the three-inlet channel used for the chemotaxis experiments. Three inlets converge into a 600 µm-wide, 100 µm-deep channel, creating three, 200 µm wide streams of cells, filtrate and attractant, respectively. The imaging window is denoted by the red box. (Inset) Diffusion of the attractant across the channel (*x* direction) creates chemical gradients to which bacteria can respond by chemotaxis. (**b**) Parallel holding chambers, 100 µm in depth and 5 mm in diameter, used for the chemokinesis experiments. Bacteria injected through the inlet were observed in the test chamber under quiescent conditions. The eight chambers on a chip allowed for parallel experiments in conjunction with the automated, programmable microscope stage that can image successive chambers efficiently and return to the precise imagine position each time. Scale bar = 5 mm.

filtrate was used both as the control attractant and as the buffer in the center inlet, to create a band separating the cells and the mucus in the microchannel (see below). Cell filtrate was obtained by passing 1.5 ml of each cell culture through a 0.2 µm syringe filter into a sterile 1.5 ml tube (Eppendorf, Hamburg, Germany). The filtrate from each of three cultures (biological replicates) at each temperature was prepared, and then the three filtrates for a given temperature were pooled to create one homogenous filtrate for each temperature.

The microfluidic devices were loaded using 23-gauge blunt-end needles (Grainger, Lake Forest, IL, USA) attached to 1 ml gastight luer tip syringes (Hamilton, Reno, NV, USA) and infused from a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA). Three syringes were filled with cells, filtrate and attractant, respectively, and connected by Tygon tubing (0.02" ID, 0.06" OD; Cole-Parmer, Vernon Hills, IL, USA) to the three inlets of the channel. Syringe contents were flowed into the device in this order to form three adjacent, 200 µm-wide bands (Figure 1a). The flow was then

stopped, resulting in the mucus chemicals diffusing across the width of the microchannel and forming a gradient in the *x* direction (Figure 1a). The pathogens' behavior in response to this gradient was recorded for 165 s with video microscopy directly downstream of the convergence point of the three inlets (Figure 1a). Negative control experiments were performed by replacing the coral mucus with filtrate. Syringes were washed with 70% ethanol and deionised water between experiments.

Three technical replicates, using the same microfluidic channel, were carried out for each biological replicate. The microchannel was freshly loaded with the three bands from the three syringe reservoirs in between each technical replicate, by flushing for 1 min at 50 µl min^{-1} and then slowing the flow to 2 µl min^{-1} before beginning a new replicate run.

Microfluidic chemokinesis experiments

Chemokinesis is defined as a change in swimming speed induced by a change in the chemical concentration in the surrounding medium, and is different from chemotaxis because it does not relate to a bias in the swimming direction, but rather to a temporal change in speed. To isolate and quantify the temperature dependence of chemokinesis in *V. coralliilyticus* in response to mucus, we used a second microfluidic device, equipped with a small (5 mm diameter, 100 µm height) holding chamber for visualization (Figure 1b). A single device contained eight individual chambers in parallel, so that multiple samples could be imaged in rapid sequence, repeatedly over time, using computer-controlled motion of the microscope stage. The mucus treatment consisted of a 1:1 mix of cells and the same mucus pools used in the chemotaxis experiments. To allow the cells enough time to respond to the chemical environment before imaging, all treatments were incubated for 8 min in 1.5 ml sterile tubes with or without mucus at their appropriate temperature (matching cell growth temperature). Thereafter, for each biological replicate at a given temperature we loaded 20 µl of the mucus-added treatment in one chamber and 20 µl of the control in the neighboring chamber, and immediately began acquisition of a 30 s long video of each treatment at 30 frames s^{-1}. We repeated the process to image a total of three technical replicates, using fresh cells each time, for each treatment, at each temperature, for all three biological replicates. The videos were processed as described below to obtain swimming speeds from cell trajectories.

Microscopy, image analysis, swimming speed quantification and statistics

All images were acquired using phase-contrast video microscopy with a 20× objective (0.45 NA) and an Andor Neo camera (6.5 µm $pixel^{-1}$; Andor, Belfast, Northern Ireland). The focal plane was always at channel mid-depth to avoid wall effects on motility. All videos were acquired at 30 frames s^{-1} to robustly capture sharp directional changes in the swimming trajectories and detect reorientations. Videos were exported in tagged image file format for analysis in MATLAB (MathWorks, Natick, MA, USA) using in-house, automated image segmentation and trajectory reconstruction software. Background subtraction and cross-correlation routines were used to detect any residual flow and to exclude non-motile cells and mucus particulates from the analysis. Individual trajectories were reconstructed from identified cell positions through subsequent frames using a particle tracking routine. Cells tracked for less than three consecutive frames were excluded from the analysis, as were trajectories with average velocity <10 µm s^{-1} (considered non-motile). From the reconstructed trajectories, we calculated the average swimming speed of each cell by averaging the instantaneous speed over the duration of that cell's trajectory. The mean speed of the population, *V,* was quantified by averaging over all trajectories at each temperature. We tested for significant differences among swimming speeds in the chemokinesis experiments using a two-tailed *t*-test to compare the mean swimming of cells at a given temperature under the two mucus conditions (with and without). The difference in speeds between those two conditions at a given temperature were also compared with the difference in speeds observed between with and without mucus conditions in the 20 °C experiments as a baseline. The significance of temperature's influence on chemotactic accumulation was tested using a one-way analysis of variance comparing the mean maximum value of cell accumulation in the mucus region ($B(x)_{Max}$; $x>500$ µm) for each temperature treatment of the chemotaxis experiments. When we found a significant effect of temperature, we removed the coolest temperature and repeated the analysis of variance to test for significant differences among the three warmer temperatures.

Results

We previously reported that *V. corallilyticus* grown at a single temperature (30 °C) employs a combination of strong chemotactic and chemokinetic behaviors in response to coral mucus (Garren *et al.*, 2014). Here, we focused on the influence of temperature on the bacterium's behavioral responses to whole coral mucus and found that chemotaxis and chemokinesis are both positively, but differentially, impacted by elevated seawater temperatures.

Temperature effect on pathogen chemotaxis

The chemotaxis experiments demonstrated that the ability of *V. coralliilyticus* cells to migrate up a gradient of coral mucus was substantially impaired at the lowest temperature (20 °C; Figures 2a and e;

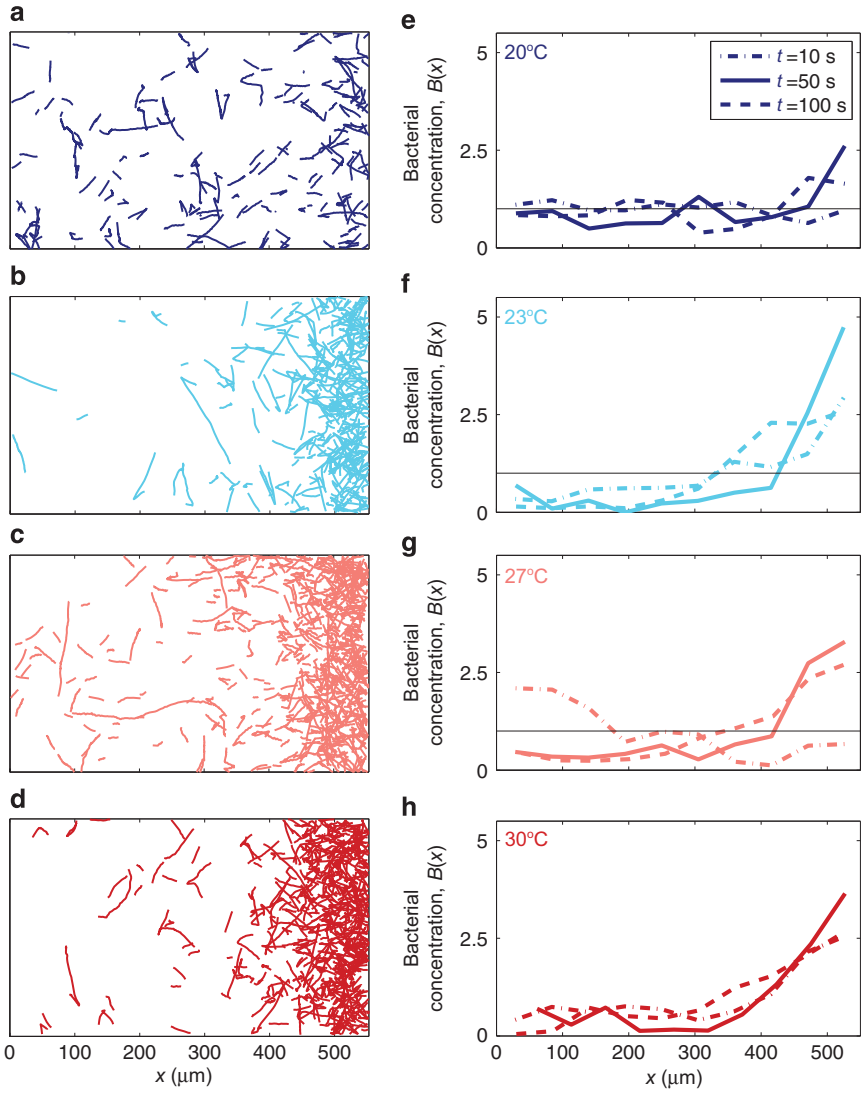

Figure 2 The strength of chemotaxis toward coral mucus by the pathogen *V. coralliilyticus* is temperature dependent. (**a–d**) Individual cell trajectories across the width of the three-inlet microchannel (Figure 1a), showing accumulation toward the side where mucus was initially injected (400 μm $< x <$ 600 μm; see Figure 1a). The trajectories shown here were acquired from 43 s to 50 s after bacteria were exposed to the mucus layer. (**e–h**) Concentration of bacteria across the channel, $B(x)$, at different times after cells were exposed to the mucus layer under diffusive gradient conditions: 10 s (dash-dotted line), 50 s (solid line) and 100 s (dashed line). The cell concentration was normalized to have a mean of one in all cases (black solid line). In all panels (**a–h**) accumulation toward the right denotes positive chemotaxis to coral mucus. Panels correspond to different temperatures at which cells were grown and assayed: 20 °C (**a**, **e**; blue), 23 °C (**b**, **f**; cyan), 27 °C (**c**, **g**; pink) and 30 °C (**d**, **h**; red). Accumulation at 20 °C is significantly weaker ($P < 0.01$) than the levels of accumulation observed in all other temperature treatments (see also Supplementary Figure S1).

Supplementary Figures S1 and S2, blue; one-way analysis of variance with $P < 0.01$). Only a weak accumulation of cells in the regions of highest mucus concentration occurred at 20 °C. In contrast, at all temperatures $\geqslant$ 23 °C, cell trajectories (Figures 2b–d) and spatial distributions (Figures 2f–h) were indicative of a significant migration toward higher coral mucus concentrations, and the maximum levels of accumulation reached were not significantly different from each other (Supplementary Figure S1 and S2; one-way analysis of variance with $P > 0.01$). For example, the bacterial concentration in the high mucus concentration region ($x = 512 \pm 12$ μm) at $t = 50$ s was 4.7-fold larger than the mean at 23 °C (Figure 2f), compared with only 2.3-fold larger than the mean at 20 °C (Figure 2e). Furthermore, a strong accumulation of bacteria in the high-mucus region persisted for considerably longer times at temperatures $\geqslant$ 23 °C (Figures 2f–h) compared with the accumulation observed at 20 °C (Figure 2e). The weaker accumulation at 20 °C was not associated with a reduction in swimming speed, which was nearly constant over the temperatures tested (Figure 3c), suggesting that the bacterium's sensing system, rather than its propulsion system, was impaired under the coolest conditions tested.

The magnitude of the chemotactic response and its temporal evolution were quantified and compared

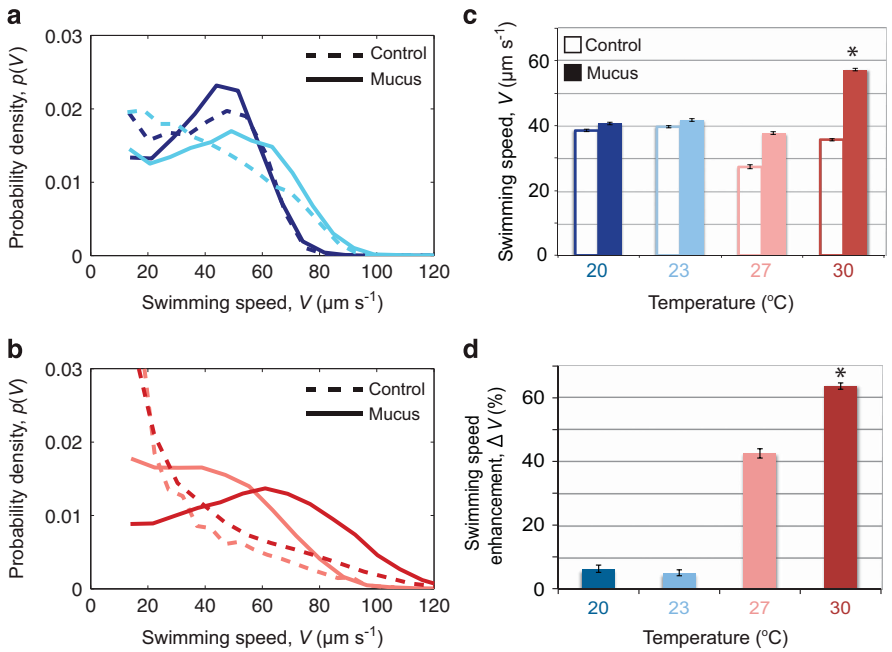

Figure 3 The strength of chemokinesis of *V. coralliilyticus* in coral mucus of uniform concentration is strongly temperature dependent. (**a** and **b**) The distribution of swimming speeds within the pathogen population for all replicates in each experiment. Curves correspond to different temperatures at which cells were grown and assayed: 20 °C (**a**, blue), 23 °C (**a**, cyan), 27 °C (**b**, pink), 30 °C (**b**, red). Solid lines are treatments with mucus and dashed lines are controls with cell filtrate in lieu of mucus. (**c**) The mean swimming speed, *V*, for cells with (solid bars) and without (open bars, controls) the addition of a uniform concentration of mucus. Cells were grown and assayed at four temperatures, indicated on the axis and by the color-coding. * denotes *P*-value <0.05 comparing the with and without mucus conditions within a given temperature. (**d**) The enhancement in the mean swimming speed of a population in the presence of coral mucus over the no-mucus control. * denotes *P*-value <0.05 when the mean is compared with the 20 °C case. Data in **c** and **d** are means of >2000 individual cell tracks for each case and the error bars denote standard errors. Experiments were performed in the circular microfluidic-holding chambers (Figure 1b).

using a normalized chemotactic index, I_C (Seymour *et al*., 2010), which measures the relative difference in cell concentration between the 200 μm-thick layer initially occupied by mucus and the 400 μm-wide region outside of that layer (Figure 1a). Absence of chemotaxis corresponds to $I_C = 0$. Some variability in the magnitude of the response among biological replicates notwithstanding, the chemotactic accumulation of cells at $\geqslant 23$ °C consistently reached $>40\%$ higher I_C values than the maximum accumulation observed in any replicate from cells at 20 °C (Supplementary Figure S2). The chemotactic response of cells at and above 23 °C was both significantly stronger ($P<0.01$) and the maximum levels of accumulation were reached more rapidly than the 20 °C treatment ($P<0.01$). For example, 20 °C cells reached their maximum state of accumulation ($I_{C,MAX} = 0.2$–0.4) in 60–70 s (Supplementary Figure S2a, b), in contrast to cells at warmer temperatures that surpassed that level of accumulation in <25 s (Supplementary Figure S2). Together these data demonstrate that, at temperatures $\geqslant 23$ °C, the chemotactic response of *V. coralliilyticus* increases by $\geqslant 60\%$ when measured as the average increase in $I_{C,MAX}$ at 23 °C, 27 °C and 30 °C over the value of $I_{C,MAX}$ at 20 °C.

Temperature effect on pathogen chemokinesis

To differentiate chemokinesis from chemotaxis at each temperature, we quantified the pathogen's response to a uniform mucus addition in the absence of a gradient. We used this approach because chemotaxis and chemokinesis can occur simultaneously, the former allowing cells to climb the gradient, the latter resulting in increases in swimming speed when substrate concentrations exceed a threshold, and both behaviors are thus folded into the quantification of chemotaxis presented above. Experiments in uniform chemical environments, without gradients, allow for the measurement of chemokinesis alone: here we quantified the absolute change in the mean swimming speed, ΔV, and the percentage change, $\Delta V_{\%}$, after the uniform addition of mucus. These experiments revealed that the swimming speed of *V. coralliilyticus* was not appreciably affected ($P>0.05$) by the addition of mucus for cells grown at 20 °C ($\Delta V = 2 \pm 4$ μm s^{-1}; $\Delta V_{\%} = 6\%$) or 23 °C ($\Delta V = 2 \pm 9$ μm s^{-1}; $\Delta V_{\%} = 5\%$) (Figures 3a, c and d). In contrast, swimming speeds appeared elevated by mucus addition in cells grown at 27 °C ($\Delta V = 10 \pm 15$ μm s^{-1}; $\Delta V_{\%} = 42\%$) and were significantly enhanced at 30 °C ($\Delta V = 23 \pm 7$ μm s^{-1}; $\Delta V_{\%} = 64\%$; $P<0.05$) (Figures 3b–d).

We emphasize that here we report not a change in swimming speed with temperature *per se*, as done previously for *E. coli* (Maeda *et al*., 1976) and other *Vibrios* (Larsen *et al*., 2004; Magariyama *et al*., 1995), but rather an increase in swimming speed elicited by mucus addition, as a function of temperature. In fact,

all swimming speed enhancements occurred against the backdrop of an otherwise temperature-independent swimming speed, which in the absence of mucus, was consistently in the range $V = 30$–40 $\mu m\,s^{-1}$ at the four temperatures tested (Figure 3c). This is in contrast to previous reports for *E. coli* (Maeda *et al.*, 1976) and two *Vibrio* species (Larsen *et al.*, 2004; Magariyama *et al.*, 1995), where speed increased monotonically with temperature over the ranges tested. Acknowledging that our current methods allow for much higher throughput analysis of individual cell behaviors than previous methods have provided, we note that temperature may have a strain-specific impact on motility, potentially with stark differences even among *Vibrios*. Our experiments demonstrate that, in addition to the advantage in gradient-sensing (chemotaxis) that the pathogen experiences at and above 23 °C, there is a second mechanism of motility enhancement in the form of increased swimming speeds (chemokinesis) as sea temperatures warm further above 30 °C.

Pathogen-intrinsic and host-derived factors combine to influence pathogen behavior

The experiments described thus far focused on the effect of temperature on the bacterial pathogen, and the mucus we used came from a uniform pool collected from coral colonies grown at 25 °C. In the natural environment, however, bacterial pathogens and corals will experience the same temperature conditions. In previous work, we have shown that the composition of coral mucus is altered when corals are subjected to elevated temperatures (Garren *et al.*, 2014). In particular, an increase in temperature from 22 °C to 31 °C resulted in a fivefold increase in the concentration of dimethylsulfoniopropionate in the coral mucus, which in turn triggered a twofold stronger chemotactic response of the pathogen (as measured by the chemotactic index) when compared with mucus from non-stressed corals (Garren *et al.*, 2014). When taken together with the results presented here, these previous findings suggest a compounding effect of temperature, which increases not only the production of the chemical signals in the mucus that attract the pathogens, but also the motility performance of the pathogen *per se*. To specifically test this combined effect of temperature on the pathogen behavior, we carried out a further set of chemokinesis (Figure 4) and chemotaxis (Figure 5) experiments where the pathogen was grown at a temperature identical or very close to that in which the corals were living when their mucus was collected. We focused on two temperatures: 22 °C, the ambient seawater temperature at the field site on Heron Island, and 30–31 °C, representative of elevated summer temperatures, for the heat-stressed mucus.

The temperature-paired pathogen-mucus experiments confirmed that chemokinesis only occurs at elevated temperatures. When 'ambient mucus'

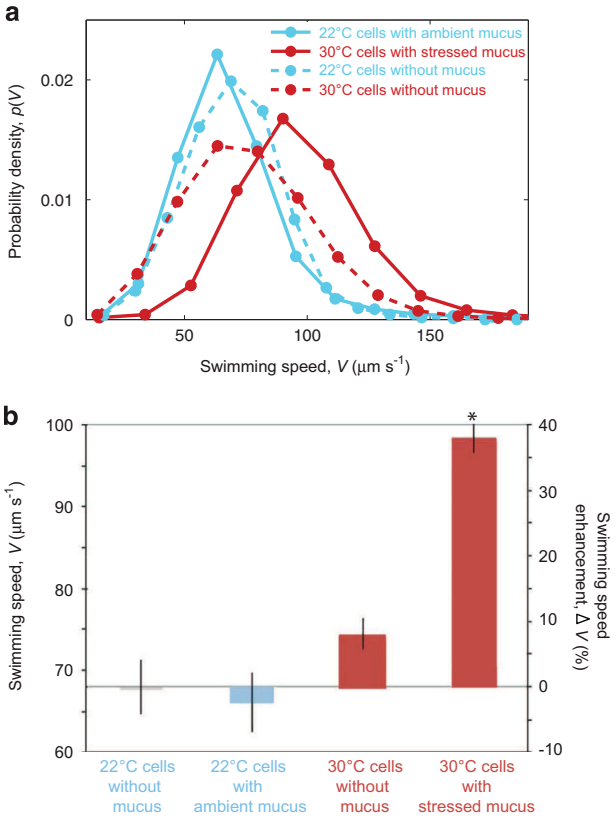

Figure 4 Chemokinetic response of *V. coralliilyticus* to mucus collected from control and heat-stressed corals. (**a**) The distribution of swimming speeds within the pathogen population showing the complete data set from all replicates of a given treatment. Pathogens grown at 22 °C were presented with a uniform concentration of mucus from healthy corals grown at ambient temperature (solid blue line), whereas pathogens grown at 30 °C were presented with a uniform concentration of mucus from heat-stressed corals (solid red line). Dashed lines represent no-mucus controls for each case. (**b**) The mean swimming speed (left axis) of all replicates for each of the four cases in panel **a**. As a second metric of the same data, values are also expressed in terms of the percent speed enhancement over the 22 °C experiment with no mucus (right axis). Error bars denote standard errors and * denotes values significantly different from both the 22 °C and 30 °C without mucus cases (P-value <0.01). Experiments were performed in the circular microfluidic-holding chambers (Figure 1b).

(that is, mucus from corals living at 22 °C when mucus was harvested) was added to 'ambient cells' (that is, cells grown at 22 °C), the swimming speed of the cells remained nearly unchanged ($\Delta V = 2 \pm 3\ \mu m\,s^{-1}$; Figure 4). In contrast, when 'heat-stressed mucus' (that is, mucus collected from heat-stressed corals) was added to 'heat-grown cells' (that is, cells grown at 30 °C), the swimming speed increased significantly by >30% ($P<0.01$, $\Delta V = 21 \pm 2\ \mu m\,s^{-1}$; Figure 4). In the absence of mucus, the speeds of ambient cells and heat-grown cells were within 10% of each other (Figure 4b). These results are consistent with the previous set of experiments testing the effect of temperature on the pathogen alone, which also showed a $\Delta V \sim 20\ \mu m\,s^{-1}$ increase in the swimming speed of 30 °C cells upon addition of mucus (Figures 3c and d). This comparison suggests that the

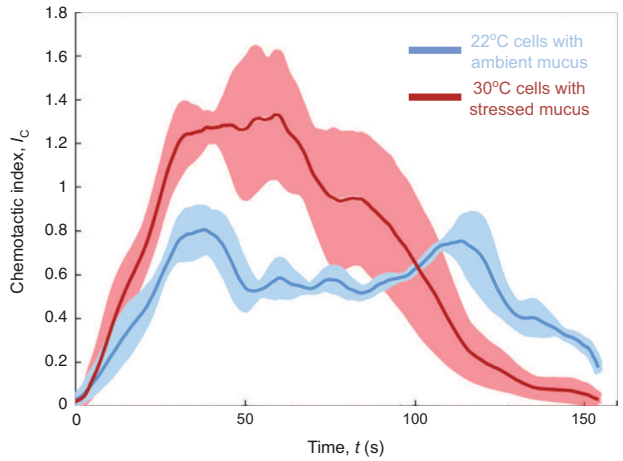

Figure 5 Chemotactic response of *V. coralliilyticus* demonstrating the combined effect of temperature on pathogen motility and host physiology. Temporal evolution of the chemotactic index, I_C, for experiments in which the pathogen was grown at the same temperatures that corals experienced when their mucus was collected: 22 °C (blue) and 30 °C (red). The chemotactic index was defined as in Supplementary Figure S2, except that here normalization occurred relative to the maximum value in the 22 °C experiments. Solid lines are means of three biological and three technical replicates. Shadings correspond to the standard error. Experiments were performed in the three-inlet channel (Figure 1a).

chemical composition of heat-stressed mucus only influences chemotaxis (Garren *et al*., 2014) and does not influence the magnitude of chemokinesis.

The most remarkable feature of *V. coralliilyticus'* host-seeking behavior in the presence of heat-stressed mucus was the marked increase in its chemotactic performance, induced by the cumulative benefit that warmer waters confer to the pathogen by intrinsically improving its motility via chemokinesis (Figure 4, Supplementary Figure S3) and by enhancing the chemical signals it follows by influencing the composition of coral mucus (Garren *et al*., 2014). This is clearly evidenced by the higher chemotactic indices reached by heat-grown cells exposed to heat-stressed mucus, compared with ambient cells exposed to ambient mucus (Figure 5). Although the pathogen does not exhibit chemokinesis at either 22 °C (Figure 4b) or 23 °C (Figure 3d), relatively strong chemotaxis occurs at both of these temperatures (with $I_{C,MAX}$ values of 0.8 and 0.9, respectively; Figure 5, Supplementary Figure S1–S3). However, unlike the nearly identical chemotactic responses observed in cells grown at 23 °C ($I_{C,MAX}$ = 0.9) and 30 °C ($I_{C,MAX}$ = 1.0) in response to 25 °C mucus (Supplementary Figure S2), cells grown at 30 °C accumulated more strongly ($I_{C,MAX}$ = 1.3; Figure 5) when responding to heat-stressed mucus than cells grown at 22 °C and exposed to ambient (22 °C) mucus ($I_{C,MAX}$ = 0.8; Figure 5). Not only will this improved chemotactic ability potentially give the bacterium an advantage in finding its host, but it could also improve cell growth by lengthening residence times in nutrient-rich (that is, mucus-rich) zones.

Discussion

Our findings suggest that under warming seawater conditions, *V. coralliilyticus* will exhibit a marked improvement in gradient-sensing abilities owing to increases in chemotactic and chemokinetic responses. These temperature-induced alterations in behavior combined with previously described changes in chemical ecology elicited in corals by heat stress (Garren *et al*., 2014) will together strongly favor the pathogen in reaching its targeted host. The cellular mechanisms underlying the switch from poor to acute chemotactic sensing and the induction of chemokinesis remain an open question. The answers will likely require a combined approach that couples genetic and transcriptomic techniques with direct observation and quantification of pathogen behaviors.

Our observations paint a distinctly different picture of the effects of increased temperature on the motility behaviors of *V. coralliilyticus*, compared with previous results using different methods for *E. coli* (Larsen *et al*., 2004), *V. alginolyticus* (Magariyama *et al*., 1995) and *V. anguillarum* (Maeda *et al*., 1976). For those species, increased temperature *per se* augmented swimming speed, whereas we observed that temperature alone has little effect on the swimming speed of *V. coralliilyticus*. Instead, we found that the change in speed upon a change in the chemical environment (chemokinesis) is strongly temperature dependent, a behavioral adaptation that to the best of our knowledge has not been reported to date for any other species.

As new tools continually improve our ability to observe bacterial behaviors, it is becoming evermore clear that bacterial motility and chemotaxis are widespread in many marine habitats (Stocker and Seymour, 2012; Son *et al*., 2015). The marine environment is a heterogeneous landscape at the microscale and a bacterium's ability to accurately navigate gradients of nutrients and infochemicals may provide it with a competitive advantage (Taylor and Stocker 2012). Chemotaxis is found governing bacteria – phytoplankton interactions (Bell and Mitchell 1972; Blackburn *et al*., 1998; Stocker and Seymour 2012), facilitating symbioses such as that between *Vibrio fischeri* and squid (Mandel *et al*., 2012), implicated in the onset of disease (Otoole *et al*., 1996; Banin *et al*., 2001; Rosenberg and Falkovitz 2004; Larsen *et al*., 2004; Meron *et al*., 2009) and common throughout coral reefs (Tout *et al*., 2015b). Within this framework, reef-building corals are becoming a focal system for studying the role of motility in bacteria–host interactions in the ocean. Corals also provide an advantage in the context of facilitating *in situ* visualization because they host an important portion of their microbiome on their external surface (Garren and Azam 2012). It is also a timely choice of from an ecological perspective because disease has recently been identified as an under-appreciated driver of the future state of reefs (Maynard *et al*., 2015).

Our observations are pertinent within the framework of changing environmental conditions that currently threaten coral reefs at a global scale. Maynard *et al.* (2015) present climate model projections for the onset of increased pathogen virulence, which was defined as a doubling in the number of months at which sea temperatures are likely to induce heightened host-seeking behaviors (that is, >historical mean monthly maximum, found experimentally to be ~27 °C). Under business as usual forecasts for greenhouse gas emissions, it is projected that pathogen virulence will increase for >40% of reefs worldwide by 2030 (Maynard *et al*., 2015). Given that all currently known putative coral pathogens are motile (Garren *et al*., 2014) and that chemotaxis is both a common feature in the reef environment (Tout *et al*., 2015a) and implicated in the success of bacterial pathogens of corals (Banin *et al*., 2001; Meron *et al*., 2009), our observations that both chemotactic and chemokinetic behaviors of *V. coralliilyticus* are influenced by temperature add to our current understanding of coral disease to suggest that motility adaptations under future climate scenarios have the potential to favor bacterial pathogens over their coral hosts (Garren *et al*., 2014; Maynard *et al*., 2015).

The goal of high-resolution observations of microbial pathogen behavior such as those reported here is to ultimately gain a better understanding of bacterial processes that drive coral disease and incorporate these into predictive models that can aid management decisions and facilitate community planning where people are dependent on reef resources. Pathogen motility is one element that may affect the outcomes of host–pathogen interactions, and disease remains an important stressor actively deteriorating reefs globally. Although the overarching picture remains complex, the ever improving techniques available to observe bacterial behaviors and elucidate the mechanistic underpinnings of disease processes at the most pertinent scales are opening the door to understand the microscale mechanisms driving ecosystem-wide patterns. In this respect, the results presented here contribute to our understanding of the potential impacts of warming seawater temperatures on bacterial behaviors that influence coral disease.

Conflict of Interest

The authors declare no conflict of interest.

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