

ORIGINAL ARTICLE

Changes in coral-associated microbial communities during a bleaching event

David Bourne¹, Yuki Iida¹, Sven Uthicke¹ and Carolyn Smith-Keune^{1,2}¹Australian Institute of Marine Science, Townsville, Queensland, Australia and ²Centre for Marine Studies, University of Queensland, St Lucia, Queensland, Australia

Environmental stressors such as increased sea surface temperatures are well-known for contributing to coral bleaching; however, the effect of increased temperatures and subsequent bleaching on coral-associated microbial communities is poorly understood. Colonies of the hard coral *Acropora millepora* were tagged on a reef flat off Magnetic Island (Great Barrier Reef) and surveyed over 2.5 years, which included a severe bleaching event in January/February 2002. Daily average water temperatures exceeded the previous 10-year average by more than 1 °C for extended periods with field-based visual surveys recording all tagged colonies displaying signs of bleaching. During the bleaching period, direct counts of coral zooxanthellae densities decreased by ~64%, before recovery to pre-bleaching levels after the thermal stress event. A subset of three tagged coral colonies were sampled through the bleaching event and changes in the microbial community elucidated. Denaturing gradient gel electrophoresis (DGGE) analysis demonstrated conserved bacterial banding profiles between the three coral colonies, confirming previous studies highlighting specific microbial associations. As coral colonies bleached, the microbial community shifted and redundancy analysis (RDA) of DGGE banding patterns revealed a correlation of increasing temperature with the appearance of *Vibrio*-affiliated sequences. Interestingly, this shift to a *Vibrio*-dominated community commenced prior to visual signs of bleaching. Clone libraries hybridized with *Vibrio*-specific oligonucleotide probes confirmed an increase in the fraction of *Vibrio*-affiliated clones during the bleaching period. Post bleaching, the coral microbial associations again shifted, returning to a profile similar to the fingerprints prior to bleaching. This provided further evidence for corals selecting and shaping their microbial partners. For non-bleached samples, a close association with *Spongiobacter*-related sequences were revealed by both clone libraries and DGGE profiling. Despite *Vibrio* species being previously implicated in bleaching of specific coral species, it is unsure if the relative increase in retrieved *Vibrio* sequences is due to bacterial infection or an opportunistic response to compromised health and changing environmental parameters of the coral host. This study provides the first molecular-based study demonstrating changes in coral-associated bacterial assemblages during a bleaching event on a natural reef system.

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Introduction

Bleaching of scleractinian corals involves the breakdown of the symbiotic association between a coral host and its photosynthetic dinoflagellates commonly known as zooxanthellae (Brown, 1997). A substantial component of a host coral nutrients is derived from the photosynthetic products produced by the zooxanthellae (Glynn, 1991). If symbiont populations are not restored within weeks or months of a bleaching event, then whole or partial

coral mortality is likely (Hoegh-Guldberg, 2004a). The reproductive capacity of corals may also be reduced for extended periods following bleaching, further negatively influencing coral populations (Szmant and Gassman, 1990). Bleaching is tightly linked to sea surface temperatures with higher than average sea surface temperatures in a given area combined with high solar radiation widely regarded as the primary cause of large-scale bleaching (Hoegh-Guldberg, 1999, 2004a,b; Jokiell and Brown, 2005). Over the last two decades, coral bleaching events have been reported with increased frequency over wide geographical scales, and, in some cases, entire reef systems have been impacted (Loya *et al.*, 2001; Hoegh-Guldberg, 2004b). Predicted ocean warming in the current century is expected to result in a further increase in the frequency of mass coral

Correspondence: D Bourne, Australian Institute of Marine Science, PMB 3, Townsville MC, Queensland 4810, Australia.
E-mail: d.bourne@aims.gov.au

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bleaching events and associated mortality (Hoegh-Guldberg, 1999, 2004a).

Pioneering works by Rosenberg and colleagues (Rosenberg and Ben-Haim, 2002; Rosenberg and Falkowitz, 2004; Rosenberg *et al.*, 2007) defined bleaching as disease in the classical sense, 'a process resulting in the tissue damage or alteration of function, producing visible physiological or microscopic symptoms'. However, debate exists as to whether coral bleaching is a direct result of environmental stress causing damage to key aspects of the zooxanthellae photosynthetic apparatus, resulting in toxic reactive oxygen by-products, and subsequent breakdown of coral–dinoflagellate symbiosis (Lesser, 1997; Jones *et al.*, 1998). An alternative view is that bleaching is the result of bacterial infection and expression of a number of virulence factors leading to the breakdown of the symbiotic association (Rosenberg and Ben-Haim, 2002; Rosenberg *et al.*, 2007). Seasonal bleaching of the Mediterranean coral *Oculina patagonica*, is the result of infection by the bacterium *Vibrio shiloi* (Kushmaro *et al.*, 1996, 1997, 1998). In this instance, the disease is the result of expression of temperature-regulated bacterial virulence genes, which produce an adhesion factor allowing attachment of the bacterium to the coral, in addition to production of extracellular toxins that inhibit photosynthesis, bleach and lyse zooxanthellae (Kushmaro *et al.*, 1998; Toren *et al.*, 1998; Rosenberg, 2004; Rosenberg and Falkowitz, 2004). In another example, bacteria-mediated bleaching and tissue lysis were demonstrated for the coral *Pocillopora damicornis*, as a direct result of infection by *Vibrio coralliilyticus* that produces a potent extracellular proteinase that may play a role in coral tissue lysis (Ben-Haim and Rosenberg, 2002; Ben-Haim *et al.*, 2003).

Recent studies indicate that despite coral-associated bacterial assemblages being highly diverse, the bacterial associations are often species specific (Rohwer *et al.*, 2002; Rohwer and Kelley, 2004; Rosenberg, 2004; Bourne and Munn, 2005). For example, Rohwer *et al.* (2002) identified 430 novel bacterial ribotypes associated with three coral species. Many appeared to have symbiotic relationships with the animal host, and Rohwer and Kelley (2004) suggested that corals may in fact harbour specific microbial communities for beneficial effect. The role these associations between bacteria and corals plays in coral health is currently not well described.

Shifts in coral-associated microbial populations may provide important indicators for environmental change and stress on reef organisms. For example, Pantos *et al.* (2003) demonstrated that shifts occurred in the bacterial community composition of the whole coral colony even when just a small part of the colony showed signs of disease. Understanding the nature of coral microbiota and how they change through time is a potentially important indicator of coral health and may provide an early

warning for changes in coral health. To date, very few microbiological studies have been performed on bleached corals. Ritchie *et al.* (1994) investigated the culturable heterotrophic bacteria of bleached and healthy *Montastrea annularis* colonies. *Vibrio* spp were never isolated from healthy corals but represented 30% of isolates from bleached corals leading the authors to speculate that a *Vibrio* spp may be responsible for the observed bleaching (Ritchie *et al.*, 1994). Further studies demonstrated that *Vibrio* spp populations tended to increase during bleaching of *M. annularis* but returned to previous levels during recovery, while populations of *Pseudomonas* spp decreased during bleaching, but also returned to previous levels during recovery (McGrath and Smith, 1999). Recent work demonstrated shifts in the heterotrophic microbial communities of stressed *Acropora palmata* corals (Ritchie, 2006). Isolates retrieved from apparently healthy colonies during a summer bleaching event were dominated by members of the genus *Vibrio* and, in addition, mucus derived from the colonies appeared to lose antibiotic activity (Ritchie, 2006). Such a reduction in antibiotic activity combined with stress of the coral may allow pathogen invasion, causing disease or contributing further to stress and bleaching.

The current study provides the first molecular-based investigation of the changes in microbial communities associated with a coral species during a bleaching event. Colonies of the ecologically important and common reef-building coral *Acropora millepora* were tagged on a reef flat (Magnetic Island, Great Barrier Reef, Australia) and sampled through a 2.5-year period, which included a prolonged bleaching event in the Austral summer of 2002. The bacterial profiles of the coral-associated microbial communities were followed with emphasis placed on investigating the relative quantity of members of the genus *Vibrio*.

Materials and methods

Field-based sampling and temperature monitoring

A total of 34 colonies of *A. millepora* were tagged on the reef flat (1.5–3 m depth) of Nelly Bay, Magnetic Island (19°10'S, 146°50'E) in October 2000. Data in this study represent samples from October 2000 through to March 2003 and incorporate samples taken during a major bleaching event on the Great Barrier Reef over the summer of 2001/2002. Tagged colonies were monitored for coral condition (visual signs of colour loss due to bleaching) and subsampled for molecular microbial analysis and zooxanthellae counts. Two branches from the centre of each colony (~2–3 cm in length) were removed during peak irradiance times (1130–1330 hours), immediately placed in plastic bags underwater, then snap frozen in liquid nitrogen at the surface before transporting back to the laboratory and stored at –80 °C.

Surface water temperatures on the Nelly Bay reef flat were obtained from October 2000 through to March 2003 using a temperature logger located approximately 50–100 m from tagged colonies. The logger was fitted with precision integrated circuit sensors (Dataflow Systems, Christchurch, New Zealand) and recorded half-hourly sea water temperatures with daily mean temperatures calculated for the duration of the monitoring period. In addition, daily average sea water temperatures from 1990 to 2000 were obtained for the Nelly Bay site, from the Great Barrier Reef Marine Park Authority (GBRMPA) water-temperature monitoring program (data supplied by R Berkelmans, Australian Institute of Marine Science). A smoothing function (a 14-day moving average) was applied to the data, allowing the daily average sea water temperature to be calculated and averaged over this 10-year period.

Bleaching assessment

The spatial extent of bleaching (white coloration) was estimated as a percentage of colony surface area on each sampling occasion. Colonies were grouped into five categories based on visual estimates of bleaching; normally pigmented, lightly bleached (<25% white), moderately bleached (25–50% white), severely bleached (>50% white) and dead (>90% dead). The validity of visual bleaching categories was confirmed by zooxanthellae density measurements for a subset of 15 colonies sampled over all time points (data not shown).

Fifteen colonies were randomly selected for quantification of bleaching parameters including zooxanthellae density (cells cm⁻²) and percentage of zooxanthellae that appeared to be in a degenerate condition. One of the two coral branches collected per colony was airbrushed with filtered sea water until no tissue remained. The tissue and filtered sea water were homogenized, the volume recorded and a subsample removed for light microscopy. Haemocytometer counts of healthy and degenerate zooxanthellae were performed under $\times 400$ magnification. Cells were considered degenerate if the cell contents were highly condensed and dark brown (Peters *et al.*, 1981). The condensed nature of the degenerate cells suggested that they represented apoptotic cells undergoing programmed cell death rather than necrotic cells, which are unlikely to be detected in air/sea water-blasted samples (Dunn *et al.*, 2002). Total densities of algal symbionts (healthy and degenerate combined) were calculated from the average of eight replicate counts per coral. Counts were corrected for subsample and total volume and normalized to coral surface area determined using paraffin wax (Stimson and Kinzie, 1991). Counts of degenerate cells were summed across eight replicate haemocytometer counts and divided by the total number of cells counted to estimate the proportion of degenerate cells. The amount of total holobiont protein (that is, host and

symbiont combined and measured as total soluble protein per mg wet weight of coral fragment; μg per mg wet weight) was determined as an additional measure of coral condition from replicate coral branches. Sampled coral fragments were ground in liquid nitrogen, and total holobiont protein was extracted from ~ 200 mg of ground material following modification of the methods of Downs *et al.* (2000). Briefly, extraction buffer (100 mM Tris pH 7.8, 20 mM EDTA, 100 mM MgCl₂, 1 mM ascorbic acid, 1 mM sodium tetraborate, 2% w/v sodium dodecyl sulphate, 3% polyvinylpyrrolidone, 50 mM dithiothreitol, 5% dimethyl sulphoxide, 10 μM mannitol, 50 μM deferoxamine mesylate and 10 $\mu\text{l ml}^{-1}$ plant protease inhibitor cocktail; Sigma-Aldrich, Sydney, Australia) was heated to 65 °C and added to ground material. Host and zooxanthellae cells were lysed at 65 °C for 10 min with regular vortexing, and insoluble material and lipids were removed by centrifugation (16 000 g, 10 min). The supernatant below the lipid layer was removed, recentrifuged and the protein content determined as described in Ghosh *et al.* (1988).

DNA extraction and purification

Three coral colonies (AM-8, AM-9, AM-28), which bleached and recovered, were selected for molecular microbial analyses. The replicate sampled coral branch was airbrushed (80 psi) with 5 ml autoclaved artificial sea water to removed coral tissue and associated microbes from the skeleton. The slurry was homogenized in a hand-held sterile glass homogeniser to break up aggregates and aliquoted into cryovials. Samples were spun at 13 000 g to pellet the cellular material, and the supernatant removed before storing the tissue pellets at -80 °C.

Coral tissue samples were resuspended in 0.5 ml sucrose extraction buffer (0.75 M sucrose, 40 mM EDTA, 50 mM Tris pH 8.3) along with sterile salmon sperm (1 μg) and the mixture left at room temperature for 5 min. Lysozyme was added (75 μl of 100 mg ml⁻¹) and incubated at 37 °C for 1 h followed by three quick freeze-thaw cycles (-80 and $+65$ °C). Sodium dodecyl sulphate was added (100 μl of 25%), mixed and incubated at 70 °C for 10 min. Samples were cooled to room temperature, and proteinase K (20 μl of 20 mg ml⁻¹) added and further incubated at 37 °C for 1 h. A further three freeze-thaw cycles were repeated before the samples were extracted in an equal volume of phenol/chloroform/iso-amyl alcohol (25:24:1). The liquid phase was removed and extracted with an equal volume of chloroform/iso-amyl alcohol (24:1). The liquid phase was removed and 50 μl of sodium acetate (3 M) added along with an equal volume of iso-propanol. DNA was pelleted (13 000 g for 15 min) and washed with 70% ethanol. DNA was electrophoresis through a 1.2% low-melting agarose gel with high-quality DNA (> 2 kb) cut from the gel and purified using the QIAquick gel extraction kit

(QIAGEN, Hilden, Germany), following the manufacturer's instructions. DNA was recovered in 50 µl sterile milli-Q water, quantified using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) and stored at -20 °C until required.

PCR amplification of 16S ribosomal RNA gene

For bacterial clone library construction, primers 27F and 1492R (Lane, 1991) were used for amplification of 16S ribosomal RNA (rRNA) genes from extracted DNA. PCR amplification of 16S rRNA genes for denaturing gradient gel electrophoresis (DGGE) was performed with primers 1055F and 1392R-GC (Ferris *et al.*, 1996). These primers amplify a 323-bp section of the 16S rRNA gene of members of the domain *Bacteria*, including the highly variable V9 region. A 40-bp GC clamp was attached to the 5' end of the 1392R primer (Muyzer *et al.*, 1993; Ferris *et al.*, 1996). All PCRs were performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) or a PE Applied Biosystems GENEAMP PCR System 9700 (Perkin Elmer, Waltham, MD, USA).

PCR was performed as follows: 0.5 µM of each primer, 100 µM of each deoxyribonucleotide triphosphate, 0.08% (w/v) bovine serum albumin, 1 × PCR buffer (Tris-Cl, KCl, (NH₄)₂SO₄, 1.5 mM MgCl₂, pH 8.7), 1.5 mM MgCl₂ (3 mM in total) and 1.25 Units of *Taq* Hot Star DNA polymerase (QIAGEN) were combined and adjusted to a final volume of 50 µl with sterile water. The temperature cycling for PCR amplification using primers 27F/1492R were 1 cycle at 95 °C for 15 min; 30 cycles at 95 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min; and one final extension at 72 °C for 10 min. PCR for DGGE profiling (primers 1055/1392R-GC) was conducted using a touchdown protocol of 1 cycle at 95 °C for 15 min, followed by 10 cycles of 94 °C for 1 min, 53 °C (decreasing in each cycle by 1 °C) for 1 min and 72 °C for 1 min. Another 20 cycles followed with 94 °C for 1 min, 43 °C for 1 min and 72 °C for 1 min as outlined in Ferris *et al.* (1996).

Clone library construction

Amplified DNA from tissue samples were ligated into a TOPO-TA Cloning vector using the protocol of the manufacturer (Invitrogen, Carlsbad, CA, USA). Ligated vector and insert were transformed into competent *Escherichia coli* cells using the methods of the manufacturer with recombinant transformations selected by blue and white screening. For each library, clones were randomly selected and grown in a microwell plate (Nunc, Roskilde, Denmark) with Luria-Bertani (LB) broth containing ampicillin (50 µg ml⁻¹) and kanamycin (20 µg ml⁻¹) for 4–6 h with shaking. All clones within the libraries were stored in microtitre plates containing glycerol at -80 °C.

Clone library restriction fragment length polymorphism analysis

The complete 16S rRNA gene insert was reamplified by PCR using the methods outlined previously with the 27F/1492R forward and reverse primers. PCR products were separately digested with 7.5 Units of the restriction endonucleases *Hha*I and *Hae*III (Promega, Madison, WI, USA) for 3 h at 37 °C. The resulting fragments were analysed on 3% high-quality ethidium bromide stained agarose gels and the restriction pattern compared. Clones having identical restriction fragment length polymorphism (RFLP) patterns were grouped into OTU (Operational Taxonomic Unit) groups. Representative clones from dominant OTU groups with two or more representative clones were sequenced.

Colony blotting and probing

A total of 96 clones within each constructed clone library were transferred to a Hybond-XL nylon membrane (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) on top of LB-ampicillin (50 µg ml⁻¹)-kanamycin (20 µg ml⁻¹) agar using sterile toothpicks and incubated overnight at 37 °C. Cell lysis on the membrane was performed as described by Sambrook *et al.* (1989). The probes used in this study to screen for *Vibrionaceae*-positive clones were *Vib*-sp1 (Franklin *et al.*, 2005) and *Vib*-GV (Giuliano *et al.*, 1999). Digoxigenin probing of membranes and probe detection were performed as described in the DIG manual (Roche Applied Science, Mannheim, Germany). Chemiluminescent detection of membranes was performed using Bio-Rad Fluor-S MultiImager and Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membrane was exposed for 15–60 min, depending on the intensity of the probe signal. For uncertain clones, a positive signal was confirmed or discarded based on sequencing of the cloned 16S rRNA gene fragment.

Denaturing gradient gel electrophoresis analysis

PCR products were separated using an INGENY phorU-2 (Ingeny International BV, Netherlands) DGGE system. Samples were loaded onto 6.5% acrylamide gels and run with 0.5 × TAE buffer (0.02 M Tris base, 0.01 M sodium acetate and 0.5 mM Na₂ EDTA; pH adjusted to 7.4) using a 50–70% linear gradient of urea and formamide. The electrophoresis was run at 60 °C, first for 20 min at 20 V and subsequently for 16 h at 75 V. After electrophoresis, the gels were stained for 30 min with SYBR Gold nucleic acid stain (Molecular Probes Inc., Eugene, OR, USA) in TAE buffer, rinsed and photographed. Distinct bands were excised from the DGGE gel and placed in 100 µl of sterile Milli-Q water overnight to elute DNA. The bands were reamplified and again run on the DGGE system to ensure purity and correct mobility of reamplified bands within the gels.

Before use as template in sequencing reactions, PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN).

Sequencing and phylogenetic analysis

All sequencing reactions were performed by MacroGen Inc. (Seoul, Korea) using BigDye Terminator cycle sequencing and products were analysed on an Automatic Sequencer model 3739xI (Applied Biosystems, Foster City, CA, USA). The 1055F oligonucleotide was used as the sequencing primer for DGGE-derived bands and the M13f primer for clone-derived sequences. Sequences were checked for chimera formation with the CHECK_CHIMERA software of the Ribosomal Database Project (Maidak *et al.*, 1996). Sequence data were aligned to the closest relative using the BLAST database algorithm (Altschul *et al.*, 1997).

Statistical analysis

For DGGE banding patterns, the software program Gel-2K developed by Svein Norland (Department of Microbiology, University of Bergen, Norway) was used to identify dominant bands within the bacterial profiles and construct a binary matrix based on presence or absence of aligned bands. On the basis of field surveys, which measure visual status of the coral colonies (see Figure 2), samples were given a score of 0 for no sign of bleaching and 1 for bleaching and included in the binary matrix. A multivariate redundancy analysis (RDA; Jongman *et al.*, 1995) was used to examine the bacterial community banding profiles within samples over the bleaching period and to illustrate relationships among community structure, average daily temperatures, mean density of zooxanthellae, percentage of degenerate zooxanthellae and total holobiont protein content. The latter four parameters were regarded as environmental parameters and z-transformed (mean = 0; s.d. = 1) prior to analysis. To further test significance of the correlations between environmental parameters, we calculated Product-Moment correlation coefficients between the environmental parameters ($n = 30$ for all comparisons). RDA and correlation analyses were conducted in S-plus 2000 (Mathsoft Inc., Cambridge, MA, USA).

Various indices and models were used to analyse the variation of microbial diversity within clone libraries (Magurran, 1988). These included the Shannon-Weaver diversity index (Shannon and Weaver, 1963), the Fisher's Alpha log series richness index (Fisher *et al.*, 1943) and Coverage (C) values (Good, 1953). OTU groupings determined from RFLP analysis of clones within the libraries were used as input for these models. The diversity of clone libraries was further investigated by rarefaction analysis (Hurlbert, 1971; Heck *et al.*, 1975; Simberloff, 1978). Rarefaction curves were produced by using the analytical approximation algorithm of

Hurlbert (1971). Calculations were performed with the freeware program aRarefact Win (Holland, 1988).

Results

Coral bleaching

Tagged *A. millepora* colonies ($n = 34$) on a reef flat at Nelly Bay (GBR) were monitored over a 2.5-year period, which included a coral bleaching event in early 2002. Average daily surface sea water temperatures during this period in relation to the daily averages over a 10-year period between 1990 and 2000 are shown in Figure 1a. The daily average temperatures in Nelly Bay exceeded the previous 10-year average by more than 1 °C for a continuous period from 6 December 2001 to 19 January 2002, including 18 consecutive days (between 27 December 2001 and 13 January 2002) with temperatures 2–3 °C above average (Figure 1b). A brief respite in water temperatures occurred with temperature less than 1 °C above average between 20 January and 30 January 2002. However, through the period of 31 January–14 February 2002, daily average temperatures were again between 1 and 2.5 °C, degrees above 1990–2000 daily averages (Figure 1b). These extended periods of elevated temperatures in the Austral summer of early 2002 were above the 31 °C threshold for which bleaching is reported for this coral species. In contrast, the temperatures measured on the reef flat in the summer periods of 2000–2001 and late 2002 to early 2003 (Figure 1a), never exceeded the 31 °C threshold for any extended period and mass bleaching of corals was not observed.

Visual signs of bleaching first became evident on the 7 January 2002 with 41% of tagged *A. millepora* colonies ($n = 14$) being categorized as lightly bleached (Figure 2). On the next sampling date (24 January 2002), all tagged colonies experienced some level of bleaching with 47% of colonies ($n = 16$) categorized as severely bleached (Figure 2). Visual bleaching coincided with a reduction in zooxanthellae density in tagged colonies (Figure 3a). Zooxanthellae density fell ~64% from 2.8×10^6 cells cm^{-2} (3 October 2001) to a low of $\sim 1 \times 10^6$ cells cm^{-2} (24 January 2002). An increase in the percentage of degenerate zooxanthellae was also observed over the same time frame (Figure 3b). Although the percentage of degenerate zooxanthellae remained low (below 4%), there was an increase from ~0.1% to ~3.8% between 3 October 2001 and 24 January 2002. A reduction in total holobiont protein also occurred during the bleaching event. Total protein dropped ~44% from 5.4 µg per mg wet weight on 3 October 2001 to ~3 µg per mg wet weight on 22 February 2002 (Figure 3c).

Microbial community changes: DGGE analysis

Three coral colonies, AM-8, AM-9 and AM-28, were chosen for analysis of their associated microbiota.

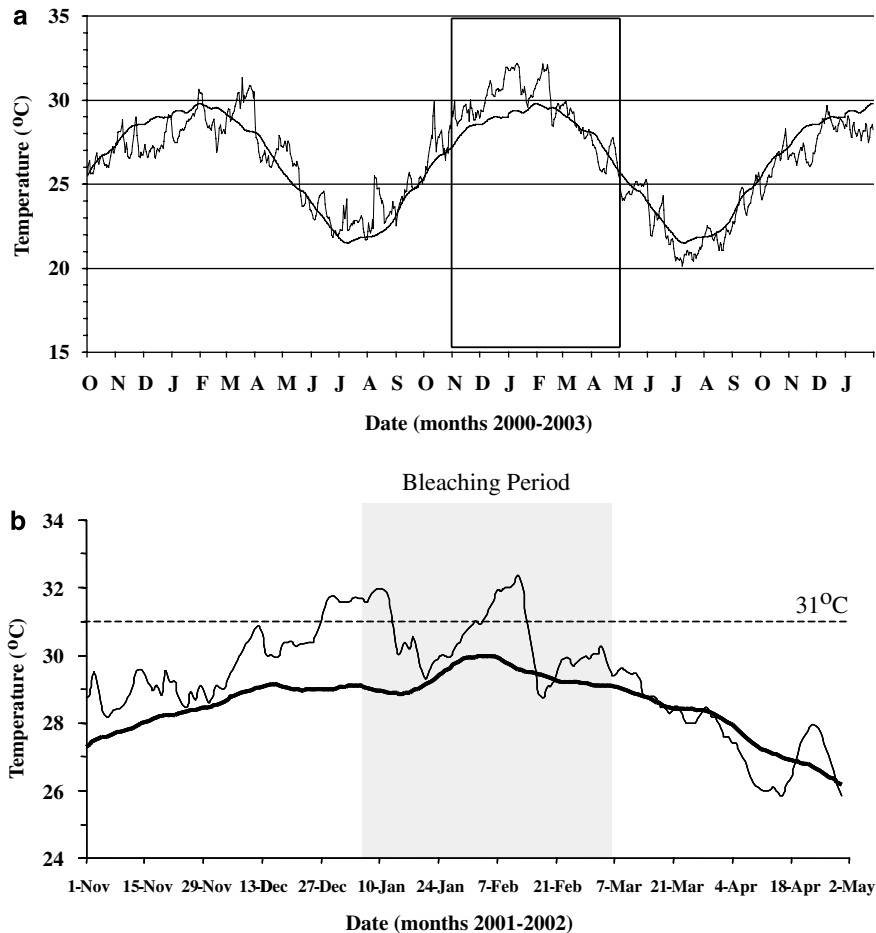


Figure 1 Nelly Bay reef flat sea water temperatures during the field study. (a) Daily average sea water temperature on Nelly Bay reef flat from October 2000 to January 2003. (b) Daily average temperature range from November 2001 to May 2002 during the coral bleaching event. The daily average sea water temperatures measured during this study are represented by the thin line (—) in both panels. The daily average sea water temperatures from 1990 to 2000 (with a 14-day smoothing function applied), derived from the GBRMPA water-temperature monitoring program is represented by the solid line (—) in both panels. The 31 °C threshold for which bleaching is reported for this coral species (Berkelmans and Willis, 1999) is represented by the dashed line in (b). Shading for (b) represents the period of the mass bleaching event on the GBR. GBR, Great Barrier Reef.

These colonies demonstrated bleaching during the warm sea water temperature periods, and DGGE analysis provided a bacterial fingerprint of the changing microbial community (10 sampling dates between 3 October 2001 and 6 June 2002). In total, eight dominant bands with distinct migration distance in the DGGE gels were successfully sequenced, and these bands affiliated with α -, β - and γ -*Proteobacteria* (Table 1). β -*Proteobacteria*-affiliated sequences represented four of the eight retrieved sequences and were related to *Delftia* sp (band 1), *Achromobacter* sp (bands 2 and 3) and *Acidovorax* sp (band 5). Band 4, which only appeared during the bleaching period, was affiliated with *Vibrio* sequences and displayed 99% sequence identity (over the 323 bp sequenced) with a *Vibrio alginolyticus* 16S rRNA gene sequence (Table 1). Band 8 affiliated with bacterial sequences previously retrieved from marine sponges with highest identity to *Spongiobacter nickelotolerans*. Band 6 interestingly affiliated with an α -*Proteobacteria*

sequence retrieved from bacterial communities associated with toxic and non-toxic dinoflagellates, while band 7 affiliated with an unidentified γ -*Proteobacteria* sequence retrieved from the Arctic marine environment.

A binary matrix representative of presence/absence of sequenced dominant DGGE bands were analysed together with environmental temperature data and coral physiological data (zooxanthellae density, percentage of degenerate zooxanthellae and total holobiont protein). RDA demonstrated a distinct shift in the coral-associated microbial community during the bleaching event (Figure 4). The first two axes of the RDA explained a high percentage of the variance (>97%) in DGGE profiles (Figure 4). The four parameters used as environmental variables (water temperature, percentage of degenerate zooxanthellae, zooxanthellae density and total holobiont protein) explained 41% of that variance. Temperature and percentage of degenerate zooxanthellae were highly correlated with each other

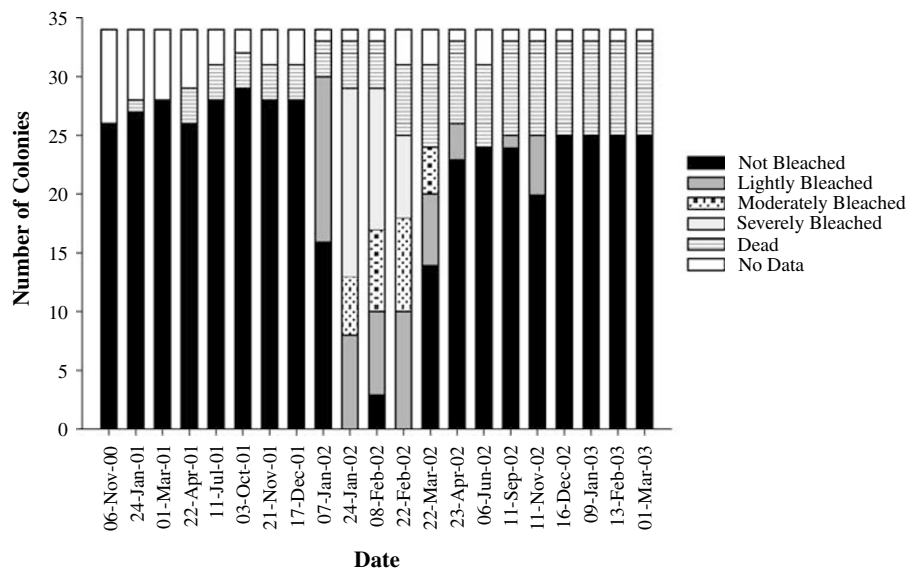


Figure 2 Temporal patterns of coral bleaching based on visible condition of *A. millepora*-tagged coral colonies ($n=34$) from Nelly Bay reef flat during October 2000–March 2003. Visual categories are represented as not bleached, lightly bleached, moderately bleached, severely bleached and dead. When colonies could not be located on the transect on a particular field survey date, no data is represented.

($r=0.564$, $P=0.001$) and negatively correlated to zooxanthellae densities (temperature: $r=-0.468$, $P=0.009$; percentage degenerate zooxanthellae: $r=-0.853$, $P<0.001$). Total holobiont protein showed no distinct correlation to the other environmental parameters (r between -0.020 and 0.315 , $P>0.05$ for each comparison).

The bacterial DGGE fingerprints of all coral samples were similar at the first pre-bleaching sampling date (3 October 2001) resulting in all profiles being grouped tightly in the RDA (Figure 4). On 21 November 2001 (sampling date 2), the bacterial profile of the AM-8 sample was identical to that of the previous month (3 October 2001), though both AM-9 and AM-28 profiles had changed. Over the next three sampling dates (3 (17 December 2001), 4 (7 January 2002) and 5 (24 January 2002)), the bacterial profiles of all coral samples shifted, with the RDA displaying a correlation of this shift with increasing sea water temperature and increase in degenerate zooxanthellae (Figure 4 profiles grouped as pre- and early bleaching period). Interestingly, this shift in the bacterial community was evident prior to the visual signs of bleaching appearing in field-based surveys. For example, bacterial profiles of AM-9 and AM-28 from 17 December 2001 (sampling date 3) clustered with profiles of colonies that displayed visual signs of bleaching on 24 January 2002 (sampling date 5). Profiles derived from coral samples in the early bleaching period from 7 to 24 January 2002 (sampling dates 4 and 5) grouped closely in the first two axes of the RDA, and these profiles were highly correlated to the appearance of the *Vibrio*-affiliated band (Figure 4).

Through sampling dates 6 (8 February 2002), 7 (22 February 2002) and 8 (22 March 2002), grouped as the late bleaching and early coral recovery phase (Figure 4), bacterial profiles shifted away from the profiles

displayed in the early bleaching period. These samples were characterized by a low amount of associated coral tissue biomass, a direct result of the loss of zooxanthellae as colonies bleached. DGGE bacterial profiles in this period also displayed a loss of the dominant *Vibrio*-affiliated band, which was reflected in the RDA plot (Figure 4). As the corals recovered post bleaching (sampling date 9 (23 April 2002) and 10 (6 June 2002)) and zooxanthellae density of coral colonies increased (Figure 3), the bacterial profiles returned to a similar profile to what they were prior to the bleaching event. This is highlighted by the tight grouping of bacterial profiles derived from samples for each coral colony on 3 October 2001 and 6 June 2002 (Figure 4; sampling dates 1 and 10). The presence of the *Spongiobacter*-affiliated band 8 was strongly correlated with the microbial profiles prior to bleaching (3 October 2001) and with the shift back to the original bacterial profiles post bleaching (6 June 2002). Bands 6 and 7 also displayed some correlation to the bacterial profiles of non-bleached samples (Figure 4).

Microbial community changes: clone library and RFLP analysis

Clone libraries derived from the AM-8 coral colony were constructed from three time points, pre- (sampling date 2 (21 November 2007): 58 clones), during (sampling date 5 (24 January 2002): 62 clones) and post- (sampling date 10 (6 June 2002): 63 clones) bleaching event. The clone library from the bleaching period (24 January 2002) contained the highest number of OTU groups (39 distinct RFLP patterns from 62 clones). Calculated diversity indices, including the Shannon–Weaver index (H'), the Fisher abundance model (α) and Simpson's

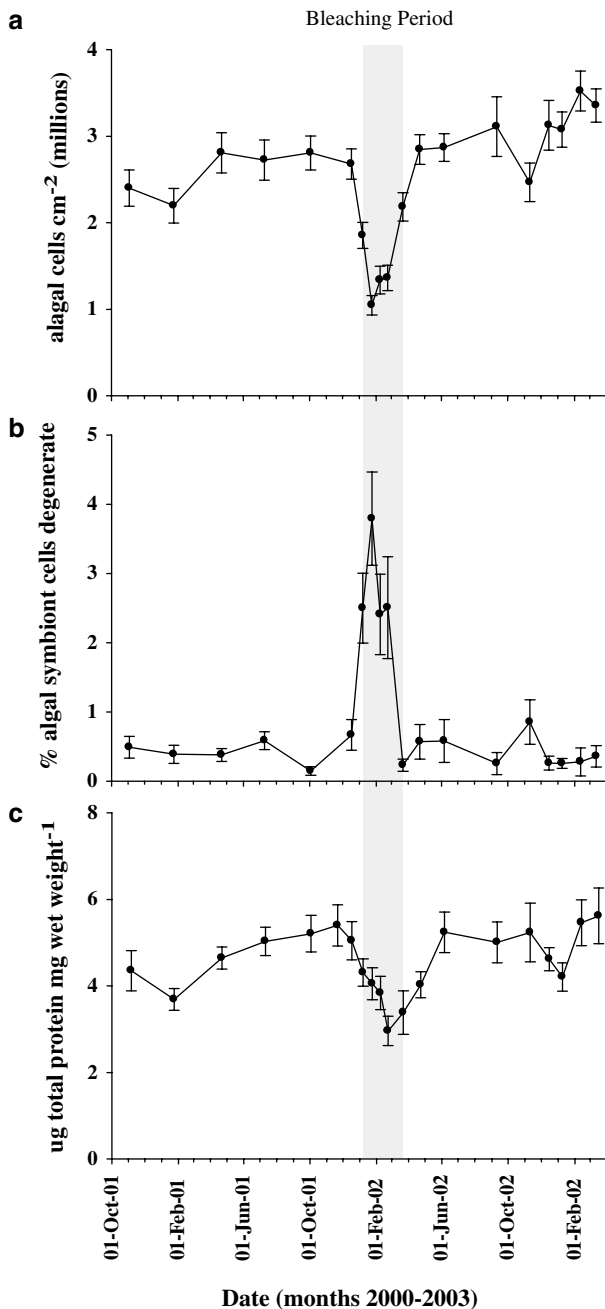


Figure 3 *A. millepora* bleaching parameters of tagged coral colonies from Nelly Bay reef flat during October 2000–March 2003. (a) Density of symbiotic algae, (b) percentage of algal symbionts degenerate in appearance and (c) µg of total protein. Shading for each graph represents the period of the mass bleaching event on the GBR. Error bars on each graph represent s.e. GBR, Great Barrier Reef.

evenness values (D) confirmed that bacterial diversity increased within the libraries during the observed bleaching period and reduced as this coral colony recovered (Table 2). As a result of the high diversity during bleaching, the coverage of this library was low at 37%. Evenness (D) values of both the pre- (21 November 2007) and post- (6 June 2002) bleaching libraries were low, indicative of dominant OTU groups within each library (Table 3). Rarefac-

tion curves did not approach an asymptote for any of the libraries (data not shown), demonstrating that sequencing of more clones would have revealed additional diversity and highlighting the high diversity of coral-associated microbial communities.

Sequencing of representative clones within each library demonstrated a change in the dominant bacterial groups during the bleaching event. The library sampled pre-bleaching (21 November 2007) was dominated by *Spongiobacter*-related sequences (OTU1 and OTU2), which represented ~41% of retrieved clones (Table 3). Interestingly, OTU1 affiliated with a sequence retrieved from a community diversity study of the coral *Muricea elongata* (GenBank accession no. DQ917877). *Vibrio*-related sequences (OTU4 and OTU9) represented the highest percentage of retrieved clones (17%) from the bleached library (24 January 2002), while *Spongiobacter*-affiliated sequences (OTU10) only represented 3% of this library. No single OTU group was dominant as reflected in the high evenness values (D) for this library (Table 2). The other clones retrieved from the bleaching library (24 January 2002) affiliated with α - and γ -proteobacterial sequences (Table 3). The library derived from the post-bleaching sample (6 June 2002) was again dominated by *Spongiobacter*-related sequences (OTU12), these being 33% of retrieved clones. The bacterial community associated with coral colony AM-8 therefore appeared to shift from one dominated by *Spongiobacter*-related sequences (21 November 2001) to a more diverse community with prominent *Vibrio*-affiliated sequences during bleaching (24 January 2001), followed by a shift back to a community dominated by *Spongiobacter*-related sequences post bleaching (6 June 2002).

Vibrio component of the bacterial clone libraries

Bacterial clone libraries were constructed for every sampling date (from 3 October 2001 through to 6 June 2002) for coral samples AM-9 and AM-28, and the libraries were screened for *Vibrionaceae* by filter hybridization using a combination of two *Vibrio*-specific probes. *Vibrio*-positive clones were first detected in the AM-28 library on 21 November 2001 and in the AM-9 library on 17 December 2001 (Figure 5). The highest fraction of *Vibrio*-positive clones was observed for both coral colonies when visual signs of bleaching were observed in field surveys (Figure 5). For the AM-9 coral sample set, the highest fraction of *Vibrio*-positive clones (~22%) was detected on 24 January 2002 (Figure 5a). For the AM-28 sample (Figure 5b), *Vibrio*-positive clones reached a peak on the 7 January 2002 sample (22%) and still represented a large proportion of the library on the 24 January 2002 sample (16%). Despite visual signs of coral bleaching being observed through the February and March sample periods (8 February–22 March 2002), the percentage of *Vibrio*-positive clones dropped

Table 1 Affiliation of DGGE bacterial sequences retrieved from coral colonies ($n = 3$) sampled through a bleaching event

Band no.	Closest relative and database accession number	Alignment (bp)	Similarity ^a (%)	Taxonomic description
Band 1	<i>Delftia acidovorans</i> strain LMG 1226 (EU024145)	321	100	β -Proteobacteria
Band 2	<i>Achromobacter</i> sp PW-1 (DQ869036)	308	99	β -Proteobacteria
Band 3	<i>Achromobacter</i> sp PW-1 (DQ869036)	317	100	β -Proteobacteria
Band 4	<i>Vibrio alginolyticus</i> strain YJ06167B (EF542800)	323	99	γ -Proteobacteria
Band 5	<i>Acidovorax</i> sp B-206 (EF596912)	317	100	β -Proteobacteria
Band 6	Marine bacterium ATAM173a_51 (AF359538)	287	100	α -Proteobacteria
Band 7	<i>Marinobacter</i> sp P11-B-10 (EU016152)	305	99	γ -Proteobacteria
Band 8	<i>Spongiobacter nickelotolerans</i> (AB205011)	323	100	γ -Proteobacteria

Abbreviation: DGGE, denaturing gradient gel electrophoresis.

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

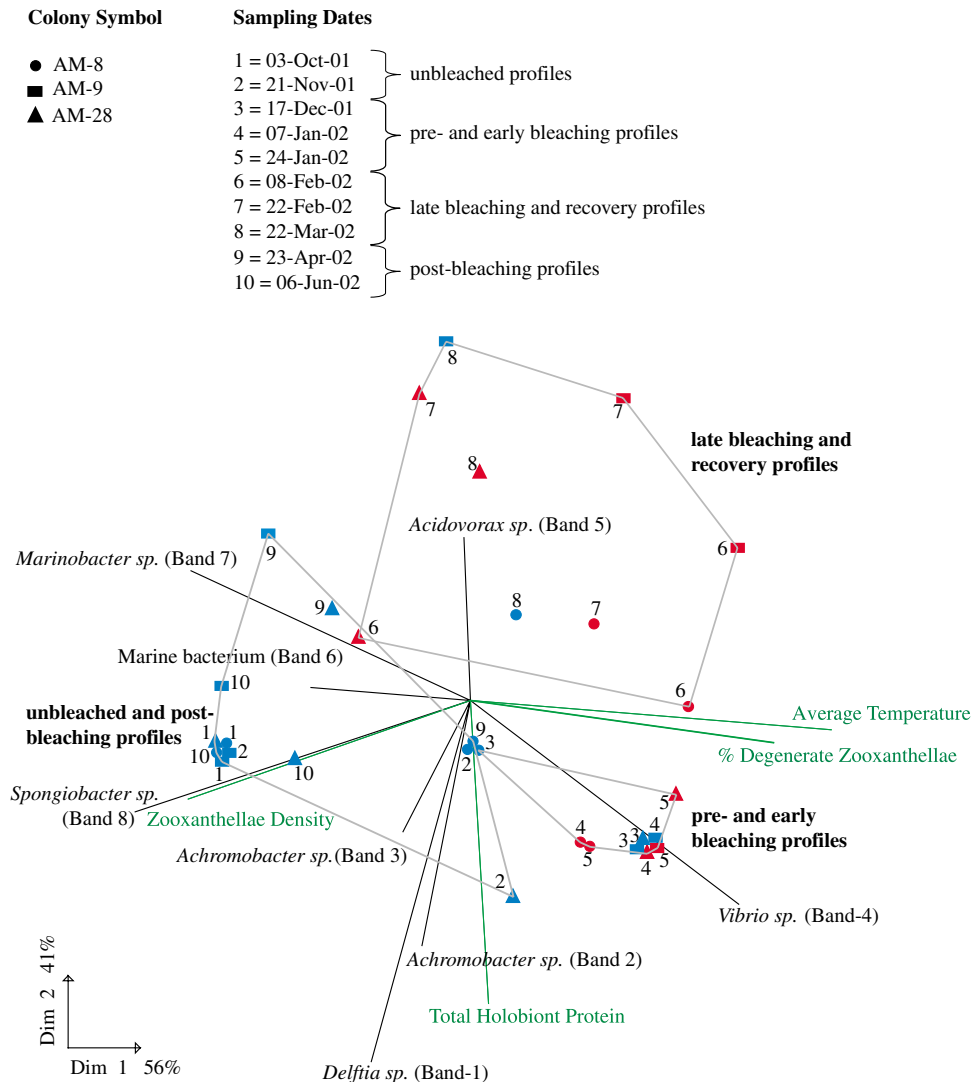


Figure 4 Biplot of a redundancy analysis (RDA) of microbial community denaturing gradient gel electrophoresis (DGGE) bacterial profiles in three coral colonies (AM-8, AM-9, AM-28) through a bleaching period from 3 October 2001 to 6 June 2002. Black lines are representative of DGGE bands incorporated in the RDA through presence/absence representation and for which sequence information was retrieved. The environmental parameter of temperature, along with coral colony measured parameters of zooxanthellae density, total holobiont protein and percentage of degenerate zooxanthellae are represented by green lines and explain 41% of the variance. All points in Figure 4 represented in red indicate samples for which the colony displayed visual signs of bleaching, while points in blue represent samples for which the colony did not display visual signs of bleaching based on visual surveys. Profiles derived from coral samples were clustered in the following groupings: unbleached and post bleaching (sampling dates 1, 2, 9, 10), pre- and early bleaching (sampling dates 3, 4, 5) and late bleaching and recovery (sampling dates 6, 7, 8). The small graph below the figure gives the amount of variance explained by the plot of the first two axes.

Table 2 Diversity indices calculated from RFLP patterns of AM-8 16S rRNA gene clone libraries

Parameters	Clone library		
	21 November 2001	24 January 2002	6 June 2002
No. of clones analysed	58	62	63
Coverage of clone libraries (%)	55	37	51
Observed no. of RFLP patterns	26	39	31
Shannon–Weaver diversity (<i>H'</i>)	2.7	3.5	3.0
Fisher's alpha (α)	18.5	45.1	24.2
Simpson's evenness (<i>D</i>)	8.2	43.0	14.2

Abbreviations: RFLP, restriction fragment length polymorphism; rRNA, ribosomal RNA.

Table 3 Affiliation of cloned bacterial sequences retrieved from coral colony AM-8 sampled through a bleaching event

Library and OTU grouping	Closest relative and database accession number	Alignment (bp)	Similarity ^a (%)	% of clone library	Taxonomic description
21 November 2001					
<i>Pre-bleaching</i>					
OTU1	Uncultured <i>Spongiobacter</i> sp clone ME82 (DQ917877)	685	98	32	γ -Proteobacteria
OTU2	<i>S. nickelotolerans</i> (AB205011)	693	99	9	γ -Proteobacteria
OTU3	<i>Brevundimonas mediterranea</i> (AJ244706)	624	99	7	α -Proteobacteria
24 January 2002					
<i>Bleaching</i>					
OTU4	<i>Vibrio costicola</i> (ATCC 35508T) (X74699)	698	99	14	γ -Proteobacteria
OTU5	<i>Stenotrophomonas maltophilia</i> (AB255384)	886	99	11	γ -Proteobacteria
OTU6	Uncultured α -proteobacterium clone FB-1_C11 (EF220223.1)	693	100	8	α -Proteobacteria
OTU7	<i>Brevundimonas vesicularis</i> (AJ007801)	905	99	6	α -Proteobacteria
OTU8	<i>Methyloarcula</i> sp BIO-204 (AJ534208)	875	99	6	α -Proteobacteria
OTU9	<i>Vibrio chagasii</i> (AJ490157)	879	99	3	γ -Proteobacteria
OTU10	Uncultured <i>Spongiobacter</i> sp clone EF0305 (EF657844)	348	91	3	γ -Proteobacteria
OTU11	<i>Serratia marcescens</i> (AB117954)	654	99	3	γ -Proteobacteria
6 June 2002					
<i>Post bleaching</i>					
OTU12	<i>Spongiobacter nickelotolerans</i> (AB205011)	700	99	33	γ -Proteobacteria
OTU13	Bacterium Ellin5130 (AY234547)	692	99	11	α -Proteobacteria
OTU14	<i>Aranicola</i> sp EP18 (AM398227)	691	99	6	γ -Proteobacteria

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

below 10% within each library after 24 January 2002. *Vibrio*-positive clones were also detected throughout the recovery and post-bleaching phases of the coral colonies, though at low levels (2–5% of clones at 6 June 2002).

Discussion

In this study, the microbial communities associated with corals were characterized and followed through a mass bleaching event that occurred on the GBR in early 2002. Previous culture-independent surveys of coral-associated bacteria have shown these communities to be extremely diverse (Rohwer *et al.*, 2001, 2002; Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Bourne and Munn, 2005). Despite highly diverse coral–bacterial associations, evidence also exists that coral species maintain a conserved microbial population (Rohwer and Kelley, 2004). For example, investigations of

Caribbean coral species by Rohwer *et al.* (2002) demonstrated that different species possessed distinct bacterial communities, though populations from the same coral species were similar even if coral individuals were separated spatially or temporally. Results from the current study support the specificity of bacterial–coral associations, with DGGE fingerprints of *A. millepora* colonies prior to a bleaching event being highly conserved between three independent coral colonies on a single reef flat on the GBR (Australia). In addition, microbial profiles of post-bleaching samples (6 June 2002) were very similar to pre-bleaching profiles (3 October 2001) providing further evidence of conserved microbial–invertebrate associations and, specifically, that species of corals maintain distinct microbial assemblages (Rohwer and Kelley, 2004). Recently, the microbiota associated with another cnidarian invertebrate, *Hydra*, was demonstrated to be conserved between samples maintained in the laboratory for >30 years and fresh samples taken

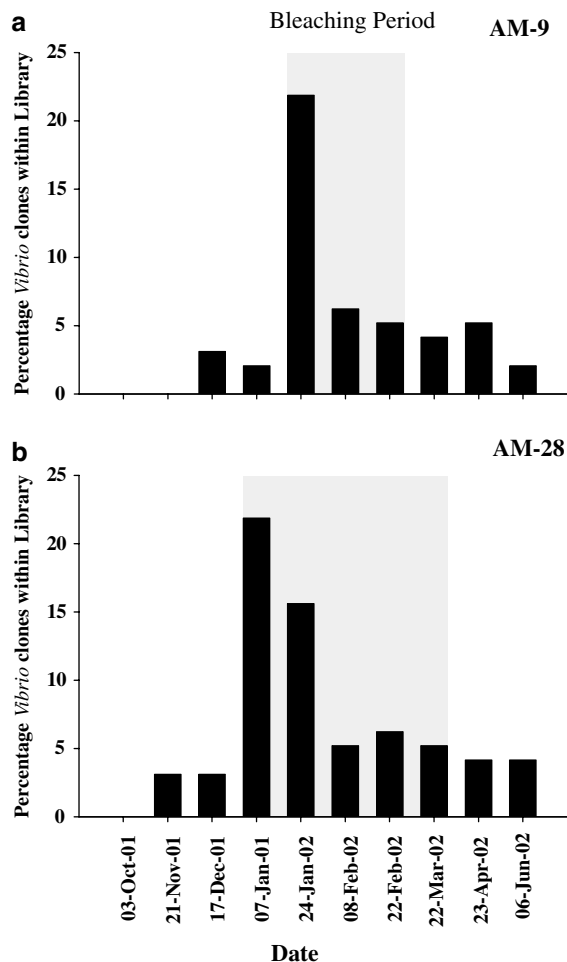


Figure 5 Percentage of *Vibrio*-affiliated clones within libraries constructed from samples of coral colonies AM-9 and AM-28 through a bleaching event (3 October 2001 to 6 June 2002). Clone libraries (100 clones) were constructed from each time period and hybridized with digoxigenin (DIG)-labelled *Vibrio*-specific oligonucleotide probes. Shading for each graph represents the period in which each individual colony recorded bleaching in field-based visual surveys.

from the marine environment (Fraune and Bosch, 2007). Such results pose interesting questions, including how such microbial communities are maintained, the potential widespread microbial mutualisms that exists in coral reef ecosystems and the important functions these may play in ecosystem health (Knowlton and Rohwer, 2003).

The dominant sequences retrieved from clone libraries both pre- and post-bleaching affiliated with *Spongiobacter* (41% and 33% of retrieved sequences, respectively), while only 3% of clones affiliated with this species from a bleached coral clone library (24 January 2002). DGGE bacterial fingerprints supported the results from the clone libraries, with profiles pre- and post bleaching characterized by the presence of a *Spongiobacter*-affiliated sequence and RDA analysis demonstrating strong correlation of DGGE profiles to this sequence. *Spongiobacter* sequences have been previously retrieved from both sponges and corals,

further supporting the notion that these animals may be selecting and shaping their microbial partners. Specific invertebrate–microbial associations are suspected to play a major role in maintaining coral health by protecting the host from invasion of potentially pathogenic microbes (Salyers and Whitt, 1994; Klaus *et al.*, 2007). It has been suggested that the resident microbial populations compete with invading microbes for nutrients and ecological niches within the mucus and tissue of the corals (Koh, 1997; Riley and Gordon, 1999; Klaus *et al.*, 2007). Evidence for these roles have been provided by a study demonstrating that bacteria within the mucus of healthy corals inhibit the growth of other bacteria 10-fold (Ritchie, 2006). *Spongiobacter*-related species may therefore be important in excluding potential invading microorganisms from the coral holobiont, and isolates derived from corals should be investigated for potential antimicrobial activity.

Elevated sea water temperatures of 1–3 °C above the long-term summer maxima, alone or in combination with increased solar irradiance, can cause large-scale coral bleaching (Brown, 1997; Hoegh-Guldberg, 2004b). The unusually warm sea temperatures on the GBR in the summer 2002 was the principal trigger for the worst mass bleaching event on record with approximately 54% of reefs experiencing some bleaching over the 2000 km length of the GBR (Berkelmans, 2002; Berkelmans *et al.*, 2004). The tagged colonies of *A. millepora* at Nelly Bay, first displayed visual signs of bleaching in early January 2002, and by 24 January 2002, all colonies displayed some level of bleaching. Visual surveys were confirmed by measurements demonstrating declines in the zooxanthellae densities and tissue biomass as well as increases in the proportion of degenerate zooxanthellae in coral colonies. RDA of DGGE bacterial fingerprints demonstrated a correlation between the observed shifts in the coral microbiota during the bleaching event with temperature and zooxanthellae density. Previous studies have demonstrated shifts in the microbial populations of diseased corals (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Pantos *et al.*, 2003; Gil-Agudelo *et al.*, 2006). However, limited studies have been performed on corals during mass coral bleaching events, and little is known of what microbial shifts occur during the bleaching and post-bleaching recovery periods in these large-scale events. In this study, DGGE bacterial fingerprints, clone library RFLP/sequencing and clone library probing analyses all demonstrated an increase in retrieval of *Vibrio*-related sequences associated with *A. millepora* colonies during a mass bleaching event. The appearance of the *Vibrio*-related band in the RDA of DGGE correlated with the shift in bacterial community structure and the increasing sea water temperatures. Clone library analysis of the AM-8 sample during the bleaching period (24 January 2002) demonstrated that *Vibrio*-related sequences represented the largest proportion of

OTUs at 17% of the library. Colony blots of clone libraries with *Vibrio*-specific probes confirmed the RFLP analysis of the AM-8 library with *Vibrio*-related clones increasing during the early bleaching period and representing approximately 22% of clones within the AM-9 and AM-28 libraries. Previous investigations of the culturable heterotrophic bacteria of seasonally bleached *M. annularis* colonies demonstrated that vibrios represented 30% of isolates from bleached corals (Ritchie *et al.*, 1994). Other culture-based studies demonstrated that *Vibrio* populations associated with corals increased during bleaching but returned to previous levels during the coral recovery period (McGrath and Smith, 1999). The molecular-based results of the present study support these earlier observations.

The shift of the bacterial population away from a stable microbiota was also supported by statistical diversity indices of clone libraries. Higher bacterial diversity was represented in a bleached sample (AM-8 sampled on 24 January 2002) when compared directly against samples from the same coral colony prior to (AM-8 sampled on 21 November 2001), and after bleaching (AM-8 sampled on 6 June 2002). Such changes in the microbial communities of corals during stressful bleaching periods is likely to change the physiological function of these communities and may therefore affect coral health. Ritchie (2006) demonstrated loss of antibiotic activity from coral mucus of *A. palmata* during a prolonged bleaching period. Without the beneficial effects of a normal microbial community and normal levels of antibiotic activity, transient bacterial populations may establish, and in worst case scenarios, coral colonies may become infected with potential pathogenic microorganisms and develop signs of disease. A small portion of the coral colonies tagged in our study suffered mortality following severe bleaching; however, the colonies for which microbial analysis was performed did not appear to become compromised by disease, and all recovered following the bleaching period.

The loss of zooxanthellae during bleaching was correlated with the loss of *Spongiobacter*-related sequences in molecular analyses. Interestingly, band 6 from the DGGE profiles was also correlated to the presence of the high numbers of zooxanthellae. Band 6 affiliated with a sequence retrieved from bacterial communities associated with toxic and non-toxic dinoflagellates (Hold *et al.*, 2001). Therefore, although temperature may affect the metabolism and diversity of the microbial community, the loss of zooxanthellae is potentially a fundamental driving parameter changing the microbiota of the corals. This is not surprising since corals reside in an environment high in levels of oxygen-free radicals and reactive oxygen species, derived from the normal photosynthetic metabolism of the symbiotic zooxanthellae (Kühl *et al.*, 1995). Reactive oxygen species acts as barrier to bacterial entry, and, therefore, loss of zooxanthellae and subsequent reduction

of reactive oxygen species would allow increased bacterial proliferation, explaining the higher bacterial diversity associated with bleached corals.

Redundancy analysis of DGGE banding patterns demonstrated that shifts in the coral microbial community occurred prior to signs of bleaching (both visual surveys and zooxanthellae density), and that these shifts correlated with the appearance of a *Vibrio*-affiliated band (Figure 4). Results from the colony blot analyses supported these results, with *Vibrio*-related clones first detected in the libraries as water temperatures started to increase, though prior to the signs of bleaching. Pantos *et al.* (2003) observed shifts in the microbial community of healthy regions of *M. annularis* coral colonies affected by white plague-like disease when compared directly to disease-free colonies. These results support the view that shifts in the conserved microbiota may serve as an early warning indicator of coral stress with microbial community changes detectable before visual signs of stress (that is, bleaching) are observed at the whole-colony level.

Previous studies have implicated *Vibrio* spp as the principal causative agent in seasonal and species-specific episodes of coral bleaching. For example, bleaching of the invasive Mediterranean coral species, *O. patagonica*, was caused by the bacterium *V. shiloi* (Kushmaro *et al.*, 1996, 1997, 1998), and bleaching and disease of the common Indo-Pacific coral species, *P. damicornis*, was attributed to the bacterium *V. coralliilyticus* (Ben-Haim and Rosenberg, 2002; Ben-Haim *et al.*, 2003). Interestingly, the highest proportion of *Vibrio*-related clones within libraries AM-9 and AM-28 were recorded on the sampling date corresponding to the first visual signs of bleaching for these respective colonies (see Figure 5). However, from the current data, it is not possible to conclude if *Vibrio* species are in fact causing disease or are opportunistically able to take advantage of the compromised nature of the coral and proliferate. Further disease progression did not occur for these corals, and they appeared to recover both visually and with zooxanthellae cell counts. Nevertheless, *Vibrio*-affiliated clones were detected in the libraries on the final sampling date of 6 June 2002, although in low numbers. Studies have previously shown that vibrios may be normal constituents of the coral microbial assemblages and, opportunistically, proliferate if holobiont health is compromised (Bourne and Munn, 2005).

In conclusion, this study was able to sample and analyse individual coral colonies through a bleaching event, and using culture independent techniques, demonstrate distinct shifts in their associated microbial communities away from conserved microbial profiles. The shifts in the microbial community with increased temperature correlated with the appearance of *Vibrio*-affiliated sequences in the samples. Recovery of the corals post bleaching resulted in the associated microbial community returning to a stable association, supporting previous

studies postulating that coral species maintain a conserved microbial population. Interestingly, microbial shifts were observed before visual signs of bleaching were recorded, providing evidence that changes in the microbial communities can act as an indicator of stress prior to the appearance of visual signs. Further studies are required to elucidate if *Vibrio* spp play a primary role in the breakdown of the symbiotic relationship between the host and its zooxanthellae partner or take advantage of the compromised health of the coral during a stressful event and increase opportunistically due to a loss of the normal microbial community and reduction in antimicrobial activity in the holobiont.

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